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EFFECTS OF OVEREXPRESSED, CONSTITUTIVELY-ACTIVE GLYCOGEN SYNTHASE ON WHOLE BODY GLUCOSE TOLERANCE AND INSULIN-STIMULATED GLUCOSE METABOLISM

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by

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Dissertation

Presented to the Faculty of the Graduate School of

The University of Texas at Austin

in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

The University of Texas at Austin

December, 2002

Acknowledgements

I would like to thank Dr. John Ivy, Zhenping Ding, Dr. Shujia Pan, Suk-Ho Lee, Ted Zderic, Mina Rathburn and Patty Coffman for helping me throughout my graduate work. I would also like to acknowledge and thank my family and friends for supporting me in all of my endeavors.

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Publication No._____

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The University of Texas at Austin, 2002

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Using the GSL3 transgenic mouse model, we have provided evidence that muscle glycogen concentration, *per se*, does not affect post-exercise insulin action *in vivo* or in muscle using an *in situ* preparation. Transgenic gastrocnemius muscle had significantly greater (7-fold) muscle glycogen concentration compared to wildtype. Glycogen concentration was unchanged 24 h following exhaustive exercise. *In vivo* insulin action during an intraperitoneal glucose tolerance test was improved 24 h following exercise regardless of the muscle glycogen concentration. Resting insulin-stimulated muscle glucose uptake was 30% lower in non-exercised transgenic versus wildtype muscle. Insulin-stimulated muscle glucose uptake was improved 24 h following exercise in

transgenic (84%) only. PI3-kinase and PKB/Akt activation was similar in non- and post-exercise wildtype and transgenic muscle. However, insulin-stimulated muscle glucose uptake and PKB/Akt activation were reduced in wildtype muscle with elevated muscle glycogen levels. Thirty minutes of contraction via *in situ* electrical stimulation of the sciatic nerve significantly reduced gastrocnemius muscle glycogen concentration in wildtype (49%) and transgenic (27%) mice although transgenic mice retained significantly more glycogen than wildtype mice. Muscle of transgenic and wildtype mice demonstrated similar pre- and post-contraction insulin-stimulated glucose uptakes. However, the percentage of [^{14}C] glucose incorporated into glycogen was greater in non-contracted (151%) and contracted transgenic muscle (157%) versus muscle of wildtype mice. PKB/Akt Ser^{473} phosphorylation was greater in transgenic mice as compared to wildtype mice post-contraction. Taken together, these results demonstrate that glycogen concentration appears to influence post-exercise muscle insulin signaling and insulin-stimulated glucose uptake in wildtype muscle when elevated above normal. However, the insulin signaling results indicate that the normal inverse relationship between muscle glycogen concentration and insulin-stimulated glucose uptake is absent in GSL3 transgenic muscle. Therefore, there may be additional mechanisms influencing the rate of insulin-stimulated muscle glucose uptake.

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CHAPTER I: INTRODUCTION

It has long been established that skeletal muscle glycogen is an important energy source during intense aerobic exercise (Ahlborg et al., 1967; Bergström et al., 1967; Hermansen et al., 1967; Hultman, 1967). Following exercise, muscle glycogen resynthesis occurs as a bi-phasic response with the first phase being insulin-independent and the second phase being insulin-dependent (Ivy, 1977; Mæhlum et al., 1977; Price et al., 1994).

Glycogen synthesis is very rapid during the insulin-independent phase but lasts only 30 to 60 minutes (Price et al., 1994). Glucose transport remains elevated immediately following cessation of exercise (Fell et al., 1982; Holloszy and Narahara, 1965; Ivy and Holloszy, 1981; Richter et al., 1982) because of an increased number of glucose transporters on the plasma membrane (Goodyear et al., 1990). The combination of a rapid glucose transport, but low glycolytic flux results in an initial elevation of glucose-6-phosphate (G-6-P) (Bloch et al., 1994). The increase in G-6-P stimulates glycogen synthase, the rate-limiting enzyme in the glycogen synthesis pathway (Bloch et al., 1994). As the residual effects of muscle contraction subside, the membrane bound GLUT4 returns to an intracellular site (Derave et al., 1999; Etgen et al., 1996; Goodyear et al., 1990) and glucose transport declines (Goodyear et al., 1990; Hespel and Richter, 1990; Holloszy and Narahara, 1965; Wallberg-Henriksson and Holloszy, 1984).

While the acute, insulin-independent effect on glucose transport rapidly declines, it is replaced by a marked increase in the sensitivity of muscle glucose transport to insulin (Cartee et al., 1989; Garetto et al., 1984; Price et al., 1994; Richter et al., 1982). This enhanced insulin action persists until the muscle glycogen level is increased to above normal (Cartee et al., 1989; Derave et al., 2000; Fell et al., 1982; Jensen et al., 1997, Kawanaka et al., 2000; Richter et al., 1984). The mechanisms responsible for the attenuation of exercise-induced insulin action are not entirely clear. However, a decline in glucose transport during post-exercise recovery appears to be inversely related to the muscle glycogen content (Cartee and Holloszy, 1990; Cartee et al., 1989; Hespel and Richter, 1990; Richter et al., 1984). Thus, it has been suggested that muscle glycogen concentration may have some control over the number of glucose transporters that can be actively associated with the plasma membrane. Recent studies utilizing the ATB-BMPA cell surface GLUT4 labeling technique have demonstrated an inverse relationship between muscle glycogen content and GLUT4 protein associated with the plasma membrane following insulin stimulation (Derave et al., 2000; Etgen et al., 1996; Kawanaka et al., 1999). Increased muscle glycogen content also lowers the activity of PKB/Akt (Derave et al., 2000; Kawanaka et al., 2000), a key insulin signaling protein involved in stimulation of GLUT4 translocation (Cheatham and Kahn, 1995). There is also an inverse relationship between glycogen concentration and the activity of glycogen synthase, the rate-limiting enzyme for glycogen synthesis (Danforth, 1965; Laurent et al., 2000).

The binding of insulin to its receptor initiates a cascade of intracellular events that ultimately results in an increase in glucose transport and metabolism (Illustration 1). Once insulin is bound to its receptor, the tyrosine kinase activity of the insulin receptor ß-subunit is activated and the insulin receptor substrate (IRS) isoforms are phosphorylated. In skeletal muscle, the predominant insulin-mediated IRS isoforms are IRS-1 and IRS-2. When phosphorylated, they act as docking proteins for downstream signaling molecules containing Src homology 2 domains including PI3-kinase (Cheatham and Kahn, 1995; Ruderman et al., 1995). PI3-kinase has been implicated in the insulin-mediated activation of PKB/Akt (Alessi et al., 1996; Cross et al., 1995) and subsequent GLUT4 translocation (Hajduch et al., 1998; Kohn et al., 1996; Tanti et al., 1997). The association between PKB/Akt activity and GLUT4 protein translocation, however, has not always been observed (Kitamura et al., 1998; Song et al., 1999). Recent research suggests that insulin activates muscle glycogen synthase in part by decreasing the activity of GSK-3 (Cross et al., 1997; Ueki et al., 1998). In skeletal muscle, this GSK-3 inactivation may be mediated by insulin-stimulated PKB/Akt activity (Cross et al., 1995). GSK-3, in turn, has been implicated in feedback inhibition of IRS-1 facilitated PI3-kinase activity (Eldar-Finkelman and Krebs, 1997; Summers et al., 1999). Thus, PKB/Akt may play a pivotal role in both insulin regulation of glucose transport and glycogen synthase activity.

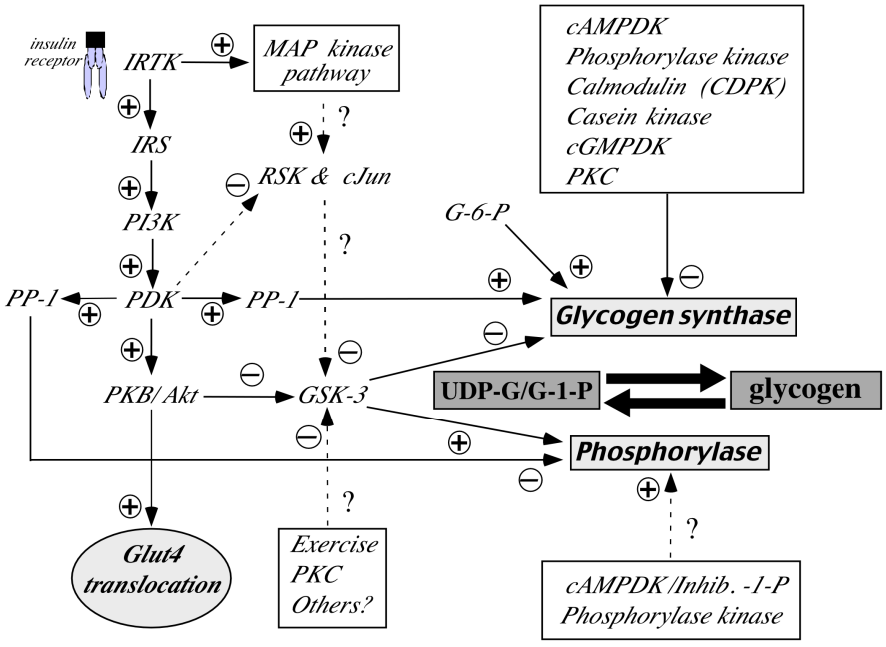


Illustration 1: Signaling proteins involved in regulation of glycogen synthase.

During post-exercise muscle glycogen resynthesis, insulin-stimulated glucose transport (Richter et al., 1984; Cartee et al., 1989) and activation of PKB/Akt by insulin (Derave et al., 2000; Kawanaka et al., 2000) remains elevated until muscle glycogen concentration is increased to above normal levels. Subsequently, PKB/Akt-mediated cell-surface GLUT4 protein (Derave et al., 2000) and glycogen synthase activity (Laurent et al., 2000; Wojitaszewski et al., 2000) are elevated until muscle glycogen levels are elevated above normal. As glycogen is supercompensated, however, the ability of insulin to activate glucose transport (Cartee et al., 1989; Derave et al., 2000; Fell et al., 1982; Jensen et al., 1997; Kawanaka et al., 2000; Richter et al., 1984), glycogen synthase (Danforth, 1965; Laurent et al., 2000) and PKB/Akt (Derave et al., 2000; Kawanaka et al.,

2000) is significantly depressed. This inhibition might occur directly via the glycogen macromolecule itself or indirectly through glycogen-mediated inhibition of PKB/Akt. Furthermore, inhibition of PKB/Akt with glycogen supercompensation could lead to reduced insulin sensitivity of IRS via disinhibition of GSK-3 (Eldar-Finkelman and Krebs, 1997; Summers et al., 1999).

Manchester et al. (1996) recently observed that glycogen concentration was markedly elevated (5-fold) in muscles of transgenic mice (GSL3) overexpressing a constitutively active form of glycogen synthase (10-fold). Azpiazu et al. (2000) extended these findings by demonstrating that glycogen synthase overexpression was associated with an increased rate of insulin-stimulated glycogen accumulation. However, *in vitro* basal and insulin-stimulated glucose uptake, as assessed by isolated muscle incubation, was not significantly different between GSL3 transgenic and wildtype mice (Azpiazu et al., 2000). These findings suggest that overexpression of glycogen synthase is sufficient to promote glycogen accumulation without reducing insulin action on muscle glucose uptake. Since the transgenic muscle did not demonstrate the classic insulin resistance typically associated with an elevated muscle glycogen level, this raises the question of whether or not insulin-stimulated glucose uptake is influenced by muscle glycogen content in this animal model.

To date, no study has assessed the importance of increased glycogen synthase activity on glycogen synthesis following exercise independent of muscle glycogen concentration. By utilizing the GSL3 transgenic mouse, which overexpresses glycogen synthase, we have investigated the effects of glycogen

synthase on post-exercise muscle glucose metabolism independent of muscle glycogen concentration. In Study 1, we determined the effects of exercise on whole body glucose tolerance and insulin-stimulated skeletal muscle glucose uptake and insulin signaling pre- and post-exercise independent of muscle glycogen concentration. Study 2 investigated the effects of elevated glycogen synthase activity on insulin-stimulated muscle glucose uptake and glycogen synthesis immediately following a lowering of initial glycogen levels by *in situ* electrical stimulation of muscle. It was hypothesized that transgenic muscle would exhibit a greater post-exercise insulin-stimulated muscle glucose uptake independent of muscle glycogen level. In other words, because of the increased activity of glycogen synthase in the muscle of these mice, enhanced post-exercise insulin action will be maintained regardless of glycogen concentration.

CHAPTER II: STATEMENT OF PURPOSE AND EXPERIMENTAL DESIGN

Statement of Purpose

STUDY 1

The purpose of Study 1 was to assess the effects of prior exercise on whole body glucose tolerance and insulin-stimulated skeletal muscle glucose uptake independent of muscle glycogen concentration. This study was designed to determine if overexpression of glycogen synthase affects: 1) whole body glucose and insulin responses to a carbohydrate challenge; 2) *in situ* skeletal muscle insulin-stimulated glucose uptake; and 3) insulin sensitivity and/or responsiveness before and after exercise independent of muscle glycogen concentration.

An acute bout of exercise has an insulin-like effect on muscle glucose transport, which rapidly reverses after exercise is stopped (Holloszy and Narahara, 1965; Wallberg-Henriksson and Holloszy, 1984). As the direct effect of muscle contraction on glucose transport is lost, it is replaced by a marked increase in the action of insulin on glucose transport and stimulation of glycogen synthase (Cartee et al., 1989; Price et al., 1994). This increase in muscle insulin action following an acute bout of exercise persists until muscle glycogen is restored to above normal levels or "supercompensates" (Richter et al., 1984; Cartee et al., 1989). The muscle glycogen levels of wildtype mice are restored and the enhanced post-exercise insulin action lost within 24 h following exhaustive

exercise (pilot data). Therefore, Study 1 was also designed to determine if overexpression of glycogen synthase: 1) affects whole body blood glucose and insulin responses to a carbohydrate challenge 24 h after exhaustive exercise independent of muscle glycogen concentration; 2) affects *in situ* skeletal muscle insulin-stimulated glucose uptake 24 h after exhaustive exercise independent of muscle glycogen concentration; and 3) affects insulin sensitivity and/or responsiveness 24 h after exhaustive exercise independent of muscle glycogen concentration.

STUDY 2

The purpose of Study 2 was to determine the effects of glycogen synthase overexpression on the post-contraction, insulin-independent phase of glycogen resynthesis. Insulin-stimulated skeletal muscle glucose uptake and glucose incorporation into glycogen were assessed under conditions in which glycogen levels were lowered by *in situ* electrical stimulation. This study was designed to determine if overexpression of glycogen synthase affects *in situ* glucose uptake and glycogen synthesis, in the absence and presence of insulin, when muscle glycogen levels are lowered by muscle contraction.

Specific Aims

STUDY 1

1) To determine whether overexpression of muscle glycogen synthase affects whole body glucose and insulin responses to a carbohydrate challenge.
2) To investigate whether overexpression of muscle glycogen synthase affects *in situ* skeletal muscle insulin-stimulated glucose uptake.
3) To determine whether overexpression of muscle glycogen synthase affects muscle insulin sensitivity and/or responsiveness.
4) To determine whether prior exercise affects whole body glucose and insulin responses to a carbohydrate challenge independent of muscle glycogen levels.
5) To investigate whether prior exercise affects *in situ* skeletal muscle insulin-stimulated glucose uptake independent of muscle glycogen levels.
6) To determine whether prior exercise affects muscle insulin sensitivity and/or responsiveness independent of muscle glycogen levels.
7) To determine whether maximizing muscle glycogen levels in wildtype mice 24 h after exhaustive exercise will result in whole body glucose and insulin responses to a carbohydrate challenge similar to those of transgenic mice.

8) To investigate whether maximizing muscle glycogen levels in wildtype mice 24 h after exhaustive exercise will result in *in situ* insulin-stimulated muscle glucose uptake similar to that of transgenic mice.
9) To determine whether maximizing muscle glycogen levels in wildtype mice 24 h after exercise will result in muscle insulin sensitivity and/or responsiveness similar to that of transgenic mice.

STUDY 2

1) To investigate whether overexpression of glycogen synthase affects *in situ* insulin-stimulated glucose uptake when muscle glycogen levels of transgenic and wildtype mice have been reduced by *in situ* electrical stimulation of muscle.
2) To assess the effects of glycogen synthase overexpression on insulin-stimulated [^{14}C] glucose incorporation into muscle glycogen following reduction of muscle glycogen by electrical stimulation of muscle.
3) To assess peak twitch tension, peak tetanic tension, and fatigability in muscles of transgenic and wildtype mice during *in situ* electrical stimulation of muscle.

Significance of Studies

Skeletal muscle is the primary tissue responsible for clearing glucose from the blood following a meal (DeFronzo et al., 1981; Baron et al., 1988). When skeletal muscle cannot remove excess glucose from the blood quickly enough to equal the glucose appearing from the gut or liver, blood glucose concentrations will rise. Muscle glycogen is the predominant storage form of glucose in the body. Insulin activates both glucose transport into the cell and the enzyme glycogen synthase, thereby affecting the initial as well as the final step in the pathway leading from extracellular glucose to muscle glycogen (Lawrence and Roach, 1997). Thus, regulation of insulin-stimulated glycogen synthesis in skeletal muscle is of particular relevance to blood glucose homeostasis. Impaired insulin-stimulated glucose uptake is associated with decreased glycogen formation and impaired glycogen synthase activity in type 2 diabetes (DeFronzo et al., 1992). In addition, glycogen synthase activity is also reduced in non-diabetic subjects with a strong family history of type 2 diabetes (DeFronzo et al., 1992).

Exercise training is beneficial in the prevention and treatment of insulin resistance (for review see Ivy et al. 1999). The actual mechanisms involved have not been completely identified but occur at the systemic, tissue, and cellular levels. Cellular adaptations responsible for the residual effects of exercise include increased skeletal muscle insulin action. The benefits of exercise on insulin action, however, start to subside rapidly once exercise ceases and are completely

lost in days. Therefore, repeated bouts of exercise of sufficient intensity and duration to lower muscle glycogen stores may benefit insulin resistant individuals.

Exercise can have both an acute and chronic effect on insulin action. With regard to the acute effect, a single bout of exercise has an insulin-like effect on muscle glucose transport, which reverses rapidly after exercise ceases (Holloszy and Narahara, 1965; Wallberg-Henriksson and Holloszy, 1984). As the direct effect on transport is lost, it is replaced by a marked increase in the action of insulin on muscle glucose transport and glycogen synthesis (Cartee et al., 1986; Garetto et al., 1984). This increase in insulin action following an acute bout of exercise persists until the muscle glycogen is restored to above normal levels or "supercompensated" (Cartee et al., 1986). Regulation of the insulin-dependent phase of post-exercise glycogen recovery is not well understood.

The relative importance of glucose transport and glycogen synthase in controlling the rate of insulin-stimulated muscle glucose uptake has been debated for years. Most evidence suggests that glucose transport is rate limiting under most conditions (Hansen et al., 1995; Ren et al., 1993; Rossetti et al., 1997; Tsao et al., 1996). However, no study has been able to demonstrate the contribution of glycogen synthase activity to glucose uptake independent of glucose transport and muscle glycogen concentration. Furthermore, the contribution of glycogen synthase independent of muscle glycogen content during the post-exercise period of enhanced insulin action has not been assessed. The transgenic mouse model used in this study has allowed investigation into the roles of increased skeletal muscle glycogen synthase activity and muscle glycogen concentration in whole

body glucose tolerance and insulin-stimulated muscle glucose uptake and glycogen synthesis. In addition, the assessment of increased glycogen synthase activity was independent of muscle glycogen and differences in glucose transporters between transgenic and wildtype mice. This novel transgenic model allowed investigation of research questions that we are unable to ascertain from rat or human experiments. This dissertation provides valuable insight into the regulation of post-exercise insulin-stimulated glucose uptake and glycogen synthesis, which, in turn, is vital in the understanding of muscle insulin resistance and type 2 diabetes mellitus.

CHAPTER III: STUDY 1

EFFECTS OF MUSCLE GLYCOGEN ON GLUCOSE TOLERANCE AND INSULIN-STIMULATED MUSCLE GLUCOSE UPTAKE PRE AND POST-EXERCISE IN TRANSGENIC MICE OVEREXPRESSING GLYCOGEN SYNTHASE

Abstract

Using a unique mouse model that overexpresses glycogen synthase, we have re-investigated the effects of muscle glycogen concentration on glucose tolerance and insulin-stimulated glucose uptake pre- and post-exercise. GSL3 transgenic (TG) mice, wildtype (WT) and glycogen supercompensated wildtype (SUPER) mice were studied using an intraperitoneal glucose tolerance test and hindlimb perfusion technique at rest or following two successive days of exhaustive exercise with carbohydrate restriction. TG gastrocnemius muscle had 7-fold greater muscle glycogen concentration compared to WT. Glycogen concentration was unchanged from pre-exercise values 24 h following the exercise/dietary manipulation in WT and TG. *In vivo* insulin action was improved following exercise regardless of the muscle glycogen concentration. Resting insulin-stimulated muscle glucose uptake was 30% lower in non-exercised TG versus WT muscle. Post-exercise insulin-stimulated glucose uptake was improved in TG (84%) but not WT. PI3-kinase and PKB/Akt activation was similar in non- and post-exercise WT and TG muscle. However, insulin-stimulated glucose uptake and activation of PKB/Akt were reduced in SUPER muscle. Using the GSL3 transgenic mouse model, we have provided evidence that muscle glycogen concentration, *per se*, does not affect post-exercise insulin action *in vivo* or in muscle using an *in situ* preparation.

Introduction

Prior exercise results in insulin-independent and insulin-dependent effects on glucose uptake in skeletal muscle. With regard to the insulin-independent effect, post-exercise muscle glucose transport is enhanced (Fell et al., 1982; Holloszy and Narahara, 1965; Ivy and Holloszy, 1981; Richter et al., 1982) due to an increased number of GLUT4 glucose transporters on the plasma membrane (Goodyear et al., 1990). As the residual effects of muscle contraction subside, the membrane-bound GLUT4 returns to an intracellular site (Derave et al., 1999; Etgen et al., 1996; Goodyear et al., 1990) and glucose transport declines (Goodyear et al., 1990; Hespel and Richter, 1990; Holloszy and Narahara, 1965; Wallberg-Henriksson and Holloszy, 1984).

While the acute, insulin-independent exercise-mediated effect on glucose transport rapidly declines, it is replaced by a marked increase in the sensitivity of muscle glucose transport to insulin (Cartee et al., 1989; Garetto et al., 1984; Price et al., 1994; Richter et al., 1982). This enhanced insulin action persists until the muscle glycogen level is increased to above normal (Cartee et al., 1989; Derave et al., 2000; Fell et al., 1982; Jensen et al., 1997, Kawanaka et al., 2000; Richter et al., 1984). The mechanisms responsible for the reversal of exercise-induced insulin action are not entirely clear. However, the decline in glucose transport during post-exercise recovery appears to be inversely related to the muscle glycogen content (Cartee and Holloszy, 1990; Cartee et al., 1989; Hespel and Richter, 1990; Richter et al., 1984). Thus, it has been suggested that glycogen

may have some control over the number of glucose transporters that can be actively associated with the plasma membrane. Recent studies utilizing the ATB-BMPA cell surface GLUT4 labeling technique have demonstrated an inverse relationship between muscle glycogen content and GLUT4 protein associated with the plasma membrane following insulin stimulation (Derave et al., 2000; Etgen et al., 1996; Kawanaka et al., 1999). Increased muscle glycogen content also lowers the activity of PKB/Akt (Derave et al., 2000; Kawanaka et al., 2000), a key insulin signaling protein involved in stimulation of GLUT4 translocation (Cheatham and Kahn, 1995). There is also an inverse relationship between glycogen concentration and the activity of glycogen synthase, the rate-limiting enzyme for glycogen synthesis (Danforth, 1965; Laurent et al., 2000).

Manchester et al. (1996) recently observed that glycogen concentration was markedly elevated (5-fold) in muscles of transgenic mice (GSL3) overexpressing a constitutively active form of glycogen synthase (10-fold). Azpiazu et al. (2000) extended these findings by demonstrating that glycogen synthase overexpression was associated with an increased rate of insulin-stimulated glycogen accumulation. However, *in vitro* basal and insulin-stimulated glucose uptake, as assessed by isolated muscle incubation, was not significantly different between GSL3 transgenic and wildtype mice. These findings suggest that overexpression of glycogen synthase is sufficient to promote glycogen accumulation without reducing insulin action on muscle glucose uptake. Since the transgenic muscle did not demonstrate the classic insulin resistance typically associated with an elevated muscle glycogen level, this raises the question of

whether or not insulin-stimulated glucose uptake is influenced by muscle glycogen content in this animal model. Using the GSL3 transgenic model, we have investigated the effects of muscle glycogen on glucose tolerance and insulin-stimulated muscle glucose uptake pre- and post-exercise. Our results suggest that the normal regulatory effect of glycogen on glucose metabolism is absent in GSL3 transgenic mice.

Methods

Experimental animals. Transgenic (GSL3 line) and non-transgenic wildtype littermates [(C57BL6 X CBA)F_1] were donated by Dr. John Lawrence, University of Virginia, Charlottesville, VA. GSL3 transgenic mice overexpress glycogen synthase [GS(2,3a)], a rabbit skeletal muscle glycogen synthase having Ser to Ala mutations at sites 2 and 3a. The generation of the transgenic line is described by Manchester et al. (1996). Transgenic expression of the constitutively active glycogen synthase is 10 times higher compared to wildtype muscle, with 5 times greater expression in fast twitch versus slow twitch muscle (Manchester et al., 1996). Upon receiving the mice, they were individually housed at The University of Texas Animal Resource Center on a 12:12 h light-dark cycle. Laboratory chow and water were provided ad libitum throughout the experiments except where noted below. The animal room temperature was maintained at 21ºC. Animals were transferred to the laboratory at least 24 h prior to the first experimental procedures following the same light cycle. The University of Texas Animal Care and Use Committee approved all procedures for this study.

Experimental procedure. GSL3 transgenic and wildtype mice (n=60) weighing 20-35 g were assigned to either non-exercised or exercised treatment groups. An additional group of wildtype mice (designated SUPER, n=12) was assigned to a glycogen supercompensation group. All mice underwent an intraperitoneal glucose tolerance test (IPGTT) followed approximately 1-2 weeks later by the hindlimb perfusion procedure. Wildtype and transgenic non-exercised groups (designated WT and TG, respectively) were allowed free access to laboratory chow and water and were otherwise restricted to normal cage activities. The wildtype and transgenic exercised groups (designated WTX and TGX, respectively) underwent two days of glycogen depleting exercise on a motorized treadmill (3 h • d^{-1}, 26 m • min^{-1}) with carbohydrate restriction between runs to limit glycogen restoration. Following the first run, mice were given 1 g of laboratory chow with lard provided *ad libitum*. Following the second run mice had free access to lard but did not receive chow. This glycogen depleting exercise/diet protocol was performed prior to both the IPGTT and the hindlimb perfusion procedure allowing glycogen restoration but not supercompensation 24 h following exercise. The wildtype SUPER mice underwent a protocol to raise muscle glycogen levels to above-normal levels. These mice performed an exhaustive run (3 h • d^{-1}, 26 m • min^{-1}) followed by 24 h of a high carbohydrate chow/table syrup mix and 15% carbohydrate (sucrose in water) solution *ad libitum*. This supercompensation procedure was performed prior to both the IPGTT and the hindlimb perfusion. Mice from all groups were fasted 6 h prior to both the IPGTT and the hindlimb perfusion.

Intraperitoneal glucose tolerance test. Mice were placed in a restrainer placed on a heating pad to maximize tail blood flow. The mice were given 1 mg • g^{-1} body weight of a 10% sterile dextrose solution by intraperitoneal injection. Blood glucose was assessed on a single drop of whole blood from the tail prior to and 30, 60, 120, and 150 min following glucose administration. In addition, approximately 0.15 ml of blood was taken prior to, 30 and 60 min following glucose administration and added to tubes containing 24 mg • ml^{-1} EDTA. Following a 10 min centrifugation in a benchtop centrifuge (3,000 *g*), the plasma was stored at -80ºC until analysis for insulin. Blood glucose was determined with the ONE TOUCH BASIC blood glucose monitoring system from Johnson & Johnson (Milpitas, CA). Validity of this blood glucose monitoring system was tested against a YSI blood glucose analyzer (Yellow Springs Instrument Company, Yellow Springs, OH). Plasma insulin was determined by radioimmunoassay (cat #: HI-11K, Linco Research, St. Charles, MO), utilizing the double antibody procedure of Morgan and Lazarow (1963).

Determination of muscle glycogen. Muscle glycogen concentration was determined by complete enzymatic degradation with amyloglucosidase (Boehringer Mannheim, Indianapolis, IN) by a modified method of Passonneau and Lauderdale (1974). A 100 µl aliquot of neutralized homogenate prepared for glucose uptake measurements was added to 250 µl of 0.3 M sodium acetate, pH 4.8 and vortexed. Then 250 µl of 0.3 M sodium acetate, pH 4.8, containing 10 mg • ml^{-1} amyloglucosidase was added and vortexed. Following an overnight incubation, 25 µl of 1 N NaOH was added and vortexed. Liberated glucose was

measured using a spectrophotometric Trinder reaction (Cat. #: 315-100, Sigma Diagnostics, Inc., St. Louis, MO). Duplicate 150 µl aliquots were placed in test tubes containing 0, 50, 100, and 200 mg • 100 ml^{-1} glucose. One milliliter of Trinder reagent was added to each tube, mixed gently and incubated for 18 min at room temperature and then read on a Beckman DU 6 spectrophotometer (Fullerton, CA) at an absorbance of 505 nm. A linear interpolation against the standard was used to determine the concentration of glucose units in the original sample. The muscle glycogen concentration was expressed as µmole glucose • g^{-1} muscle.

Measurement of glycogen synthase activity. Glycogen synthase was measured by direct incorporation of [U-^{14}C] UDP-1-glucose (ICN Biochemicals, Costa Mesa, CA) into glycogen with several concentrations of G-6-P (0.01, 0.5, 1.5, and 25 mM) as previously described (Sherman et al., 1988). Total activity was defined as that measured in the presence of 25 mM G-6-P. Fractional velocities of submaximal (0.5 and 1.5 mM G-6-P) to that of maximal (25 mM) G-6-P glycogen synthase activity (expressed as percentages) as well as -/+ G-6-P activity ratios were also calculated. The activity ratio was calculated as the enzyme activity measured in the presence of 0.01 mM G-6-P divided by the total activity.

Hindlimb perfusion. Mice were anesthetized with an intraperitoneal injection of pentobarbital sodium (6.5 mg • 100 g^{-1} body weight) following a 6 h fast and kept warm by performing the surgery on a heating pad. The surgical technique was similar to that described previously (Brozinick et al., 1996; Ivy et

al., 1983). Major vessels branching from the abdominal aorta and vena cava except the common iliac artery and iliac vein were ligated. Heparin (100 U) was injected into the inferior vena cava just inferior to the diaphragm. Following the heparin injection, the descending aorta and inferior vena cava were cannulated as previously described (Ivy et al., 1983). Catheters were placed in line with the non-circulating hindlimb perfusion apparatus providing a 37ºC, continuously gassed (95% O_2/5% CO_2) perfusate flow of 1.8 ml • min^{-1}. Both hindlimbs were perfused during the equilibrium (10 min) and tracer (20 min) periods. Perfusates consisted of 6% bovine serum albumin (BSA) in Krebs-Henseleit buffer (KHB, pH 7.4) with 0.2 mM pyruvate. During the tracer period, the perfusate contained 6 mM [1,2-^{3}H] 2-deoxyglucose (7.5 µCi • $mmol^{-1}$; ICN Biochemicals), 2 mM [U-^{14}C] mannitol (60 µCi • $mmol^{-1}$; ICN Biochemicals) and the appropriate insulin (Humulin R-100, Eli Lilly, Indianapolis, IN) concentration (0.2 mU • ml^{-1} or 10 mU • ml^{-1}). Immediately following the perfusion period, both gastrocnemii were dissected from the hindlimbs, frozen with Wollenberg tongs cooled in liquid N_2 and stored frozen (-80ºC) until further analysis.

Determination of 2-Deoxyglucose Uptake. Freeze-clamped gastrocnemius muscle from the perfused hindlimbs were sectioned and weighed frozen. A 60-100 mg piece of mixed-fiber muscle was dissolved in 1 ml 1 N KOH by incubating for 15 min at 65ºC, mixed, and incubated an additional 5 min at 65ºC. An equal volume of 1 N HCl was added to the digested samples, mixed, and aliquots of the neutralized samples counted for [^{3}H] and [^{14}C] DPM (Beckman LS 6000SC). Muscle 2-deoxyglucose uptake was calculated from the specific activity

of the original perfusate after subtracting out the appropriate volume for extracellular space as determined from radiolabelled mannitol in the muscle sample. Mixed gastrocnemius was used because it has been shown to exhibit a high transgene expression but contains similar GLUT4 content in GSL3 transgenic and non-transgenic littermates (Manchester et al., 1996).

GLUT4 protein concentration. Frozen muscle was homogenized (1:20 wt:vol) in HES buffer containing 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffer, 1 mM EDTA, and 250 mM sucrose (HEPES, pH 7.4). Samples were homogenized at 15,000 *g* for 20 min at 4°C and protein determinations were performed on each homogenate using the method of Bradford (1976). A 75 µg protein aliquot of each supernatant sample and a mouse heart standard were subjected to SDS-PAGE on a 12.5% polyacrylamide resolving gel. A mouse heart supernatant standard (16 µl) was run on each gel. A Miniprotean II dual slab cell (BioRad, Richmond, CA) was used to perform the electrophoresis. Samples were transferred from the resolving gel onto polyvinylidene diflouride (PVDF) sheets (BioRad). The transferred PVDF membranes were blocked for 1 h by incubation in 5% nonfat dry milk (Carnation, Los Angeles, CA) in TTBS (0.06% Tween-20, 20 mM Tris, 500 mM NaCl, pH 7.5) at room temperature. Following a rinse using TTBS, the membranes were incubated with GLUT4 rabbit anti-rat antibody (donated by Dr. Samuel Cushman, NIH, Bethesda, MD). After two additional 5 min rinses in TTBS, the membranes were incubated with horseradish peroxidase-conjugated donkey anti-rabbit antibody (Amersham, Arlington Heights, IL) for one hour. The membranes were

then washed in sacosyl buffer (50 mM Tris Base, 1 M NaCl, O.4% w/v N-laurylsarcosine, 5 mM EDTA, pH 7.5) three times to decrease non-specific binding. After the blotting procedure, antibody-bound protein was visualized using an ECL Western blot detection kit (Amersham) according to the manufacturer's instructions. GLUT4 concentration was expressed as percent heart standard.

Hexokinase activity. For assessment of hexokinase activity, HES homogenates were centrifuged in a benchtop centrifuge at 3,000 g for 15 min. Hexokinase activity was measured on the supernatant using the spectrophotometric determination of glucose-6-phosphate as described by Uyeda and Racker (1965).

Insulin Signaling Proteins. Insulin-stimulated muscle samples were prepared as described by Kawanaka et al. (2000). Muscle pieces (30 mg) were homogenized in ice-cold buffer containing 50 mM HEPES (pH 7.4), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1.0 mM EDTA, 10 mM $Na_4P_2O_7$, 100 mM NaF, 2.0 mM Na_3VO_4, aprotinin (10 µg • ml^{-1}), leupeptin (10 µg • ml^{-1}), pepstatin (0.5 µg • ml^{-1}), 1% Igepal and 2 mM phenylmethylsulfonyl fluoride. Homogentates for IRS-1-associated PI3-kinase activity were incubated with end-over-end rotation at 4ºC for 60 min and then centrifuged at 200,000 *g* for 50 min at 4ºC. IRS-1-associtated PI3-kinase was immunoprecipitated using a 2 µg • $µl^{-1}$ protein sample as previously described (Christ et al., 2001) using an anti-IRS-1 antibody (2 µg, Upstate Biotechnology Inc., Lake Placid, NY) for 2 h at 4ºC, followed by an overnight protein A-Sepharose (Sigma) incubation (4ºC). The

immunoprecipitates were successively washed prior to *in vitro* phosphorylation of phosphatidylinositol. Sonicated phosphatidylinositol (Avanti Polar Lipids, Pelman, AL) was added to each sample and the PI3-kinase reaction started by addition of 10 µl of 50 mM $MgCl_2$, 250 µM [γ-^{32}P]-ATP (0.5 µCi • ml^{-1}, Amersham) in a buffer consisting of 20 mM HEPES (pH 7.4), 0.4 mM EGTA, and 0.4 mM Na_2PO_4 and stopped by addition of 6 M HCl. Lipids were extracted by chloroform-methanol (1:1) and applied to a thin layer chromatography plate (Silica gel 60, Whatman, Hillsboro, OR). The plates were developed (60 min) in running solvent ($CHCl_3$:MeOH:H_2O:NH_4OH, 60:47:11.3:2). Radioactivity of TLC spots for standards and samples was quantified via scintillation counting for ^{32}P (Beckman LS 6000SC).

For quantification of phosphorylated PKB/Akt-1α, aliquots of the 200,000 *g* supernatant were treated with 2x Laemmli sample buffer containing 100 mM dithiothreitol and boiled for 5 min. Samples (80 µg protein) were subjected to SDS-PAGE (10% resolving gel) and transferred to nitrocellulose membranes. An insulin-stimulated mouse gastrocnemius muscle standard (80 µg) was run on each gel. The membranes were blocked in 5% nonfat dry milk in TBS containing 0.1% Tween 10 (pH 7.5) for 1 h. Following a rinse in 0.1% Tween 10, the membranes were incubated with sheep antiphospho-PKB/Akt-1 (Ser^{473}) antibody (Upstate Biotechnology Inc.) for 4 h. Three isoforms of PKB/Akt have been identified. The primary insulin-stimulated isoform in rodent muscle is PKB/Akt-1α (Turinsky and Damrau-Abney, 1999; Walker et al., 1998). In addition, insulin-stimulated PKB/Akt-1α activity was inversely related to skeletal muscle glycogen content 18

h post-exercise in the study by Derave et al. (2000). Therefore, PKB/Akt-1α is was the isoform assessed in the present study. Membranes were rinsed in 0.1% Tween 10 and incubated with horseradish peroxidase-conjugated rabbit anti-sheep IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) for 60 min. Antibody-bound protein was visualized using an ECL Western blot detection kit (Amersham) according to the manufacturer's instructions. Phosphorylated PKB/Akt-1α of samples was expressed relative to the insulin-stimulated mouse standard.

Total PKB/Akt-1α protein content was assessed on nitrocellulose membranes used for phosphorylated PKB/Akt-1α. Membranes were stripped of antibodies by incubation with stripping buffer containing 100 mM 2-mercaptoethanol, 2% sodium dodecyl sulfate, 62.5 mM Tris HCl (pH 6.7) for 30 min. Membranes were then washed three times for 15 min in TTBS buffer. Membranes were blocked overnight, washed three more times for 15 min in TTBS, and incubated with sheep anti-Akt/PKB-1α (1:1000 vol/vol) (Upstate Biotechnology Inc.) for 4 h with gentle agitation. Following three more TTBS washes, antibody-bound protein was visualized using ECL (Amersham) and total PKB/Akt-1α was expressed relative to the insulin-stimulated mouse standard.

Statistics. One-way analysis of variance was performed on muscle glycogen, glycogen synthase, GLUT4, hexokinase, and total PKB/Akt. Repeated measures analyzing treatment versus time was performed for IPGTT blood glucose and plasma insulin responses. An additional one-way analysis of variance comparing groups was performed for individual IPGTT time points. For glucose

uptake, IRS-1-associated PI3-kinase activity and phosphorylated PKB/Akt-1α, one-way analysis of variance was performed for experimental groups within low and high insulin levels. Post hoc tests were performed using Fishers's Protected LSD and differences were considered statistically significant if $P \leq 0.05$.

Results

IPGTT responses. During an intraperitoneal glucose tolerance test, blood glucose responses were not different between TG and WT mice (Figure 3.1). Resting blood glucose concentrations for TGX and WTX mice were approximately 50% of those observed in the non-exercised groups. Prior exercise resulted in a lower blood glucose response in WTX compared to WT. This exercise effect was not observed in transgenic mice. In addition, the blood glucose response in TGX was significantly higher than WTX. This was due to TGX blood glucose concentrations that were 20-25% greater than the WTX at 30 and 60 min following the glucose injection. Glycogen supercompensation did not result in altered glucose tolerance, as SUPER and WTX blood glucose responses were similar.

Plasma insulin responses to the carbohydrate challenge were similar in TG and WT mice (Figure 3.2). Prior exercise resulted in a significantly lower plasma insulin response as responses in TGX, WTX and SUPER mice were significantly lower than the non-exercised groups prior to, 30 and 60 min following the glucose injection. However, exercise elicited a greater effect in WTX compared to TGX as plasma insulin was significantly higher in TGX versus WTX mice at 30 min.

Muscle glycogen. Gastrocnemius glycogen concentration was 7-fold greater in TG compared to WT (Figure 3.3). Following 2 days of exercise and dietary manipulation, WTX and TGX gastrocnemius glycogen concentrations were similar to pre-exercise values. Subjecting wildtype mice to a 24 h glycogen supercompensation protocol following exhaustive exercise (SUPER) increased muscle glycogen 3.6-fold compared to WT and WTX. However, SUPER muscle glycogen levels remained markedly lower than the transgenic groups.

Glycogen synthase activity. Glycogen synthase fractional velocities at 1.5 and 0.5 mM G-6-P and -/+ G-6-P activity ratios were similar in all groups (Table 3.1). However, there were considerable differences in terms of absolute glycogen synthase activity, expressed in nmol • g^{-1} wet weight • min^{-1}, is presented in Figure 3.4. Total activity of glycogen synthase as well as allosteric activation at submaximal G-6-P concentrations (1.5 and 0.5 mM) were significantly greater in TG and TGX compared to WT, WTX, and SUPER (Figure 3.4). This was true for either perfusion insulin concentration. In TG, TGX, and WTX, glycogen synthase total activity was approximately 120% greater following high insulin perfusion compared to the low insulin perfusion. Allosteric activation by submaximal G-6-P concentrations (1.5 and 0.5 mM) following perfusion with high insulin was greater in TG compared to WT and SUPER. Prior exercise did not significantly affect insulin-stimulated total glycogen synthase activity in either transgenic or wildtype muscle. SUPER glycogen synthase total activity was not different from WT or WTX following perfusion with either insulin concentration.

2-Deoxyglucose uptake. Muscle glucose uptake was 30-36% lower in TG versus WT muscle at both perfusion insulin concentrations (Figure 3.5). However, there were no differences in glucose uptake between WTX and TGX at either insulin concentration. During perfusion with low insulin, glucose uptake remained similar for WT and WTX but glucose uptake of TGX was significantly higher than TG. A similar response was found during perfusion with the high insulin concentration. Glucose uptake in TGX was approximately 84% higher than TG during both low and high insulin perfusions. SUPER muscle glucose uptake was 45% lower than WT during the low insulin perfusion. This trend was also observed during the high insulin perfusion although the difference (15%) was not statistically significant. SUPER muscle glucose uptake was 51% of WTX during the low insulin perfusion and 73% of WTX during perfusion with a high insulin concentration.

GLUT4 protein concentration and hexokinase activity. Gastrocnemius muscle GLUT4 protein concentrations were similar in WT, WTX and TG (Figure 3.6). However, gastrocnemius GLUT4 concentrations of TGX and SUPER were significantly higher than WT. Hexokinase activity was similar between WT, WTX, TGX, and SUPER (Figure 3.7). TG hexokinase activity was significantly greater than WTX and SUPER.

Insulin signaling proteins. IRS-1-associated PI3-kinase activities in muscles from WT and TG groups were not significantly different although activity in transgenic muscle tended to be higher (Figure 3.8). During perfusion with the low insulin concentration, exercise did not affect PI3-kinase activity in

either genotype. However, following the high insulin perfusion, prior exercise was associated with a significantly greater insulin-stimulated activation of PI3-kinase in wildtype. This was not the case in transgenic muscle as TG and TGX PI3-kinase activities were similar following the high insulin perfusion. SUPER PI3-kinase activity was only assessed following the high insulin perfusion. SUPER PI3-kinase activity was lower but not significantly different from WT. However, PI3-kinase activity was significantly lower in SUPER compared to WTX.

Total PKB/Akt protein concentration was similar in wildtype and transgenic muscle (Figure 3.9). Exercise, *per se*, had no effect on total PKB/Akt protein (i.e. WTX similar to WT and TGX similar to TG). Total PKB/Akt protein of SUPER, however, was significantly higher than that found for WT, WTX, and TGX groups. Perfusion insulin concentration did not affect total PKB/Akt protein concentration (data not shown).

Insulin-stimulated PKB/Akt (Ser^{473}) phosphorylation was similar between WT and TG as well as between WTX and TGX for both perfusion insulin concentrations (Figure 3.10). Previous exercise increased PKB/Akt phosphorylation in both genotypes following the low insulin perfusion (103% in wildtype and 50% in transgenic). The slight increase in PKB/Akt phosphorylation following the high insulin perfusion was not significantly different in either genotype. Phosphorylated PKB/Akt in SUPER muscle was significantly lower than WTX following the low insulin perfusion as well as the high insulin perfusion. In addition, SUPER muscle phosphorylated PKB/Akt was significantly

lower than WT following the high insulin perfusion and although this trend was also observed during the low insulin perfusion, it was not statistically significant.

Discussion

Acute exercise is associated with a marked increase in the sensitivity of muscle glucose transport and glycogen synthesis to insulin during the insulin-dependent phase of post-exercise glycogen recovery (Cartee et al., 1989; Garetto et al., 1984; Holloszy and Narahara, 1965; Price et al., 1994; Ren et al., 1994; Richter et al., 1984). Attenuation of improved insulin action during post-exercise recovery appears to be related to muscle glycogen levels (Cartee and Holloszy, 1990; Cartee et al., 1989; Hespel and Richter, 1990; Richter et al., 1984). The mechanisms responsible for the reversal of exercise-induced insulin action are not entirely clear. However, elevated muscle glycogen concentrations are associated with decreased insulin-stimulated cell surface GLUT4 transporter protein (Derave et al., 2000; Etgen et al., 1996; Kawanaka et al., 1999) and PKB/Akt activity (Derave et al., 2000; Kawanaka et al., 2000), a key insulin signaling protein involved in stimulation of GLUT4 translocation (Cheatham and Kahn, 1995). Using the GSL3 transgenic mouse model, we have re-examined the effects of muscle glycogen concentration on glucose tolerance and insulin-stimulated muscle glucose uptake pre- and post-exercise. We have provided evidence that muscle glycogen concentration, *per se*, may not affect post-exercise insulin action *in vivo* or in muscle using an *in situ* preparation.

In the present study, IPGTT glucose responses were similar in non-exercised wildtype and GSL3 transgenic mice suggesting that muscle glycogen concentration does not affect glucose tolerance. In addition, glucose tolerance was improved in exercised wildtype although muscle glycogen concentrations of exercised and non-exercised wildtype were similar. In contrast, post-exercise glucose tolerance was not improved in mice with above normal muscle glycogen concentrations (i.e., GSL3 transgenic and glycogen supercompensated wildtype). Prior exercise, however, resulted in a much lower plasma insulin response regardless of muscle glycogen concentration. A lower insulin response despite a normal or improved glucose response is indicative of enhanced insulin action in tissues responsible for glucose disposal. Thus, unlike glucose tolerance, post-exercise *in vivo* insulin action was improved regardless of the muscle glycogen concentration. This suggests that the effect of acute exercise on *in vivo* insulin action is independent of the muscle glycogen concentration.

Azpiazu et al. (2000) demonstrated that glycogen synthase overexpression was associated with an increased rate of insulin-stimulated glycogen accumulation while basal and insulin-stimulated glucose uptake remained similar to wildtype mice. These *in vitro* findings suggest that overexpression of glycogen synthase is sufficient to promote glycogen accumulation but does not improve insulin action on glucose uptake in muscle. In the present study, insulin-stimulated muscle glucose uptake was lower in non-exercised GSL3 transgenic versus wildtype muscle. This finding is in contrast with those of Azpiazu et al. (2000) but agrees with several studies demonstrating an inverse relationship

between muscle glycogen concentration and insulin-stimulated muscle glucose uptake (Fell et al., 1982; Hespel and Richter, 1990; Jensen et al., 1997). Further support for this inverse relationship is evident when comparing our post-exercise insulin-stimulated muscle glucose uptake results from exercised wildtype and glycogen supercompensated wildtype. When glycogen levels had recovered following exercise in wildtype muscle, insulin-stimulated glucose uptake was normal. However, when muscle glycogen is elevated to above normal levels, glucose uptake is significantly reduced compared to non- and post-exercised wildtype treatments.

The inverse relationship between muscle glycogen concentration and insulin-stimulated muscle glucose uptake was not observed in post-exercise GSL3 transgenic muscle. The effect of glycogen on post-exercise, insulin-stimulated glucose uptake was not consistent with that observed in wildtype mice. GSL3 transgenic muscle demonstrated an increase in post-exercise insulin-stimulated glucose uptake independent of muscle glycogen concentration. Thus, muscle glycogen does not appear to be a regulator of insulin-stimulated muscle glucose uptake in GSL3 transgenic mice post-exercise. Therefore, the post-exercise insulin resistance normally associated with above normal muscle glycogen levels is eliminated in this model. Why post-exercise insulin action is maintained in GSL3 transgenic muscle is not immediately clear. In the present study, we have assessed several key sites of insulin action to address this question.

The binding of insulin to its receptor initiates a cascade of intracellular events that ultimately results in an increase in glucose transport and metabolism.

Once insulin is bound to its receptor, the tyrosine kinase activity of the insulin receptor ß-subunit is activated and the insulin receptor substrate (IRS) isoforms are phosphorylated. In skeletal muscle, the predominant insulin-mediated IRS isoforms are IRS-1 and IRS-2. When phosphorylated, they act as docking proteins for downstream signaling molecules containing Src homology 2 domains including PI3-kinase (Cheatham and Kahn, 1995; Ruderman et al., 1995). PI3-kinase has been implicated in the insulin-mediated activation of PKB/Akt (Alessi et al., 1996; Cross et al., 1995) and subsequent GLUT4 translocation (Hajduch et al., 1998; Kohn et al., 1996; Tanti et al., 1997). The association between PKB/Akt activity and GLUT4 protein translocation, however, has not always been observed (Kitamura et al., 1998; Song et al., 1999). Recent research suggests that insulin activates muscle glycogen synthase in part by decreasing the activity of GSK-3 (Cross et al., 1997; Ueki et al., 1998). In skeletal muscle, this GSK-3 inactivation may be mediated by insulin-stimulated PKB/Akt activity (Cross et al., 1995). GSK-3, in turn, has been implicated in feedback inhibition of IRS-1 facilitated PI3-kinase activity (Eldar-Finkelman and Krebs, 1997; Summers et al., 1999). Thus, PKB/Akt may play a pivotal role in both insulin regulation of glucose transport and glycogen synthase activity.

During post-exercise muscle glycogen resynthesis, insulin-stimulated glucose transport (Richter et al., 1984; Cartee et al., 1989) and activation of PKB/Akt by insulin (Derave et al., 2000; Kawanaka et al., 2000) remains elevated until muscle glycogen concentration is increased to above normal levels. Subsequently, PKB/Akt-mediated cell-surface GLUT4 protein (Derave et al.,

2000) and glycogen synthase activity (Laurent et al., 2000; Wojitaszewski et al., 2000) are elevated until muscle glycogen levels are elevated above normal. As glycogen is supercompensated, however, the ability of insulin to activate glucose transport (Cartee et al., 1989; Derave et al., 2000; Fell et al., 1982; Jensen et al., 1997; Kawanaka et al., 2000; Richter et al., 1984), glycogen synthase (Danforth, 1965; Laurent et al., 2000) and PKB/Akt (Derave et al., 2000; Kawanaka et al., 2000) is significantly depressed. This inhibition might occur directly via the glycogen macromolecule itself or indirectly through glycogen-mediated inhibition of PKB/Akt. Furthermore, inhibition of PKB/Akt with glycogen supercompensation could lead to reduced insulin sensitivity of IRS via disinhibition of GSK-3 (Eldar-Finkelman and Krebs, 1997; Summers et al., 1999).

In the present study, prior exercise was associated with a significantly greater maximal insulin-stimulated IRS-1-associated PI3-kinase activity in wildtype muscle. This finding is in agreement with a recent study by Chibalin et al. (2000) in which insulin-stimulated IRS-1-associated PI3-kinase activity was increased 2.6-fold in rat muscle 16 h following acute exercise. An increase in insulin-stimulated PI3-kinase activity was also observed with submaximal insulin although this increase did not reach statistical significance. The post-exercise activation of PKB/Akt with insulin followed a similar pattern as that observed with PI3-kinase activity. In wildtype muscle, submaximal insulin-stimulated PKB/Akt phosphorylation was doubled and maximal insulin-stimulated PKB/Akt phosphorylation was increased slightly (29%), but not significantly, following exercise. However, despite increased insulin activation of PI3-kinase and

PKB/Akt, post-exercise insulin-stimulated glucose uptake was not improved at either perfusion insulin concentration. Taken together, these results demonstrate a disassociation between insulin signaling and glucose uptake following exercise in wildtype mice when muscle glycogen levels have recovered.

When wildtype muscle glycogen concentration was elevated above normal levels (i.e., supercompensated), the exercise effect of increased maximal insulin-stimulated IRS-1-associated PI3-kinase activity was abolished. This finding is in contrast to recent evidence suggesting that muscle glycogen content does not affect the insulin-mediated activation of this kinase (Derave et al., 2000; Kawanaka et al., 2000). In addition, phosphorylated PKB/Akt in glycogen supercompensated muscle was significantly lower than exercised wildtype following both perfusion insulin concentrations. The decrease in IRS-1-associated PI3-kinase activity and corresponding reduction in the phosphorylation of PKB/Akt were associated with impaired insulin-stimulated glucose uptake in glycogen supercompensated muscle. The impairment of insulin-stimulated glucose uptake that was observed in glycogen supercompensated wildtype was not due to a lower total GLUT4 protein content or hexokinase activity. On the contrary, total GLUT4 protein was significantly elevated in glycogen supercompensated muscle. Taken together, these results suggest that glycogen appears to influence post-exercise muscle insulin signaling and insulin-stimulated glucose uptake but only when elevated to above normal levels in wildtype mice.

The normal suppression of insulin signaling associated with an elevated muscle glycogen concentration was disrupted in muscle overexpressing a

constitutively active glycogen synthase. In non-exercised GSL3 transgenic muscle, insulin-stimulated glucose uptake was lower than that observed in non-exercised wildtype despite slightly higher PI3-kinase activity and PKB/Akt phosphorylation in the transgenic muscle. The differences in insulin-stimulated glucose uptake observed between non-exercised wildtype and GSL3 transgenic could not be accounted for by differences in GLUT4 protein or hexokinase activity. This finding suggests that an alternative mechanism, other than reduced PKB/Akt activity, may exist for a reduced insulin-stimulated glucose uptake during conditions of elevated muscle glycogen.

Following exercise, insulin-stimulated muscle glucose uptake was higher in exercised GSL3 transgenic compared to exercised wildtype and glycogen supercompensated treatments. This post-exercise increase in GSL3 transgenic insulin-stimulated glucose uptake occurred despite markedly elevated muscle glycogen. Although a significant increase in IRS-1-associated PI3-kinase activity was not observed in GSL3 transgenic muscle as in wildtype, IRS-1-associated PI3-kinase activity was greater in GSL3 transgenic muscle compared to glycogen supercompensated wildtype. PKB/Akt activation (phosphorylated to total PKB/Akt ratio) was similar in exercised GSL3 transgenic and exercised wildtype. Again, exercised GSL3 transgenic PKB/Akt activation was higher than glycogen supercompensated wildtype muscle. Our GSL3 transgenic findings contrast the inhibition of insulin signaling and glucose uptake observed in the glycogen supercompensated wildtype treatment. Furthermore, the GSL3 transgenic results differ from previous studies (Derave et al., 2000; Kawanaka et al., 2000)

demonstrating an inverse relationship between muscle glycogen concentration and PKB/Akt phosphorylation. The finding that insulin-stimulated muscle glucose uptake was higher in GSL3 transgenic versus glycogen supercompensated wildtype suggest that the putative association between glycogen levels and glucose uptake is absent in the transgenic model. Why resting and post-exercise PKB/Akt phosphorylation remains highly active in GSL3 transgenic muscle despite elevated muscle glycogen remains unclear. However, this may be related to the constitutively active glycogen synthase. Furthermore, the influence of glycogen on PKB/Akt may be due to the ability of glycogen to depress glycogen synthase activity. This may represent a putative, yet unknown, association between the activation states of glycogen synthase and PKB/Akt, both of which are normally inhibited when muscle glycogen has been elevated above normal.

Recently, it was demonstrated that immediately following exhaustive exercise, skeletal muscle GLUT4 mRNA is elevated (Kuo et al. 1999a; Ren et al., 1994). However, once carbohydrate is consumed, the GLUT4 mRNA concentration declines while total GLUT4 protein is increased (Kuo et al. 1999b). Kuo et al. (1999b) suggested that there is an increased GLUT4 mRNA translation efficiency with carbohydrate supplementation following exercise. Our finding that GLUT4 protein was significantly higher in glycogen supercompensated muscle than exercised wildtype or transgenic mice further support this theory. The glycogen supercompensated mice were provided a high carbohydrate diet immediately following exercise while carbohydrate was restricted in the exercised wildtype and transgenic treatments. In addition total PKB/Akt protein in glycogen

supercompensated muscle was significantly higher than in exercised wildtype and GSL3 transgenic suggesting that control of PKB/Akt protein expression is similar to that of GLUT4.

In summary, we have determined the effects of glycogen synthase over expression on post-exercise muscle glucose metabolism independent of muscle glycogen concentration. Using the GSL3 transgenic mouse model, we have provided evidence that muscle glycogen concentration, *per se*, does not affect post-exercise insulin action *in vivo* or in muscle using an *in situ* preparation. Following exercise, GSL3 transgenic muscle exhibits greater insulin action and insulin-stimulated muscle glucose uptake independent of muscle glycogen content relative to wildtype muscle. The increase in insulin-stimulated muscle glucose uptake following exercise in GSL3 transgenic muscle could not be accounted for by increases in GLUT4 protein or hexokinase activity. Glycogen concentration appears to influence post-exercise muscle insulin signaling and insulin-stimulated glucose uptake in wildtype muscle when elevated above normal. However, our insulin signaling results support the possibility that the normal mechanisms responsible for the inverse relationship between muscle glycogen concentration and glucose uptake may be absent in GSL3 transgenic muscle. This may represent a putative, yet unknown, association between the activation states of glycogen synthase and PKB/Akt, both of which are normally inhibited when muscle glycogen has been elevated above normal. In addition, overexpression of glycogen synthase may facilitate a repartitioning of intracellular glucose

intermediates towards glycogen synthesis as opposed to the glycolytic, hexosamine, and oxidative pathways.

Table 3.1 Gastrocnemius muscle fractional velocity at 1.5 mM and 0.5 mM G-6-P; and -/+ G-6-P activity ratio following hindlimb perfusion with either low (0.2 mU • ml^{-1}) or high (10 mU • ml^{-1}) insulin concentration. Fractional velocities are expressed as percentages. Groups are wildtype (WT, N=4), transgenic (TG, N=4), exercised wildtype (WTX, N=4), exercised transgenic (TGX, N=5), and wildtype exercised/supercompensated (SUPER, N=5).

Group	Perfusion Insulin	Fract. Vel. (1.5 mM G-6-P)	Fract. Vel. (0.5 mM G-6-P)	Activity Ratio (-/+ G-6-P)
WT	low	32.7 ± 18.1	7.0 ± 2.6	0.03 ± 0.014
	high	19.0 ± 2.9	7.8 ± 0.8	0.04 ± 0.013
TG	low	17.1 ± 2.7	5.4 ± 0.9	0.02 ± 0.003
	high	26.0 ± 4.7	6.9 ± 1.3	0.02 ± 0.005
WTX	low	19.9 ± 2.5	7.3 ± 0.8	0.04 ± 0.010
	high	22.3 ± 6.8	8.1 ± 2.1	0.03 ± 0.014
TGX	low	19.0 ± 2.3	5.1 ± 0.6	0.01 ± 0.002
	high	25.3 ± 4.0	6.8 ± 1.3	0.01 ± 0.002
SUPER	low	16.8 ± 2.9	11.6 ± 3.2	0.06 ± 0.033
	high	18.6 ± 3.9	12.5 ± 5.4	0.09 ± 0.045

Figure 3.1. Blood glucose response to a 1 mg • g BW^{-1} intraperitoneal glucose tolerance test. Groups are wildtype (WT, N=10), transgenic (TG, N=12), exercised wildtype (WTX, N=12), exercised transgenic (TGX, N=13), and wildtype exercised/supercompensated (SUPER, N=8). Group means ± SEM presented. †$P < 0.05$, non-exercised vs. exercised within each genotype; ‡$P < 0.05$, TG vs. WTX; *$P < 0.05$, WT vs. TGX, #$P < 0.05$, WTX vs. SUPER; §$P < 0.05$, TGX vs. SUPER; %$P < 0.05$, TG vs. SUPER; @$P < 0.05$, WTX vs. TGX.

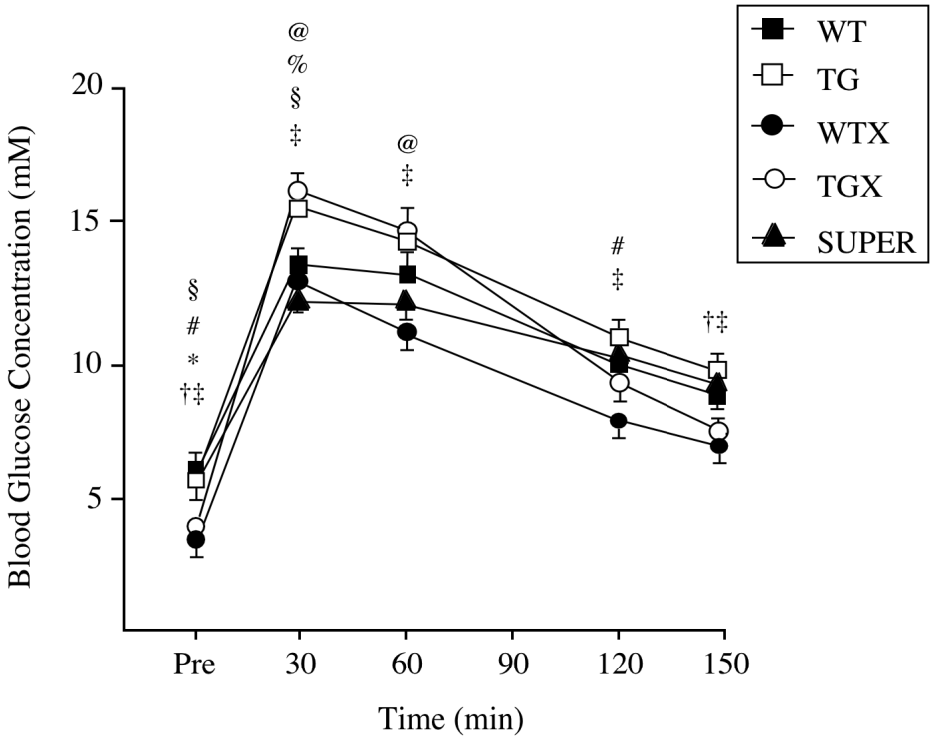


Figure 3.2. Plasma insulin response to a 1 mg • g BW^{-1} intraperitoneal glucose tolerance test. Groups are wildtype (WT, N=10), transgenic (TG, N=12), exercised wildtype (WTX, N=12), exercised transgenic (TGX, N=13), and wildtype exercised/supercompensated (SUPER, N=8). Group means ± SEM presented. †$P < 0.05$, non-exercised vs. exercised within each genotype; ‡$P < 0.05$, non-exercised groups vs. SUPER; *$P < 0.05$, WTX vs. TGX.

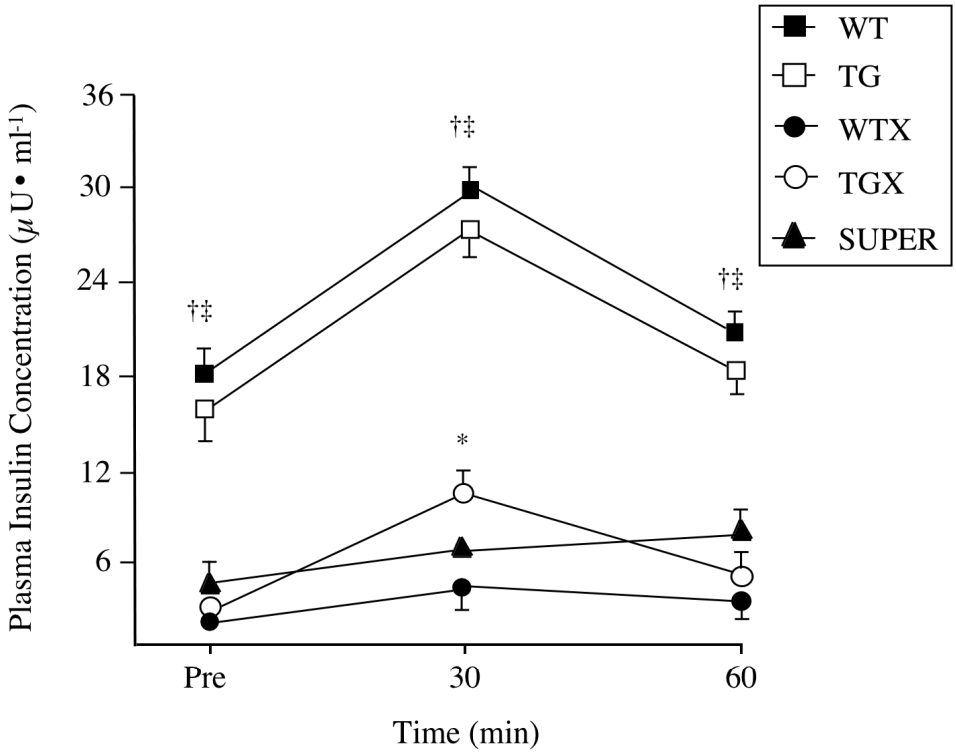


Figure 3.3. Muscle glycogen content in mixed gastrocnemius muscle from GSL3 transgenic and wildtype mice. Groups are wildtype (WT, N=13), transgenic (TG, N=14), exercised wildtype (WTX, N=13), exercised transgenic (TGX, N=15), and wildtype exercised/supercompensated (SUPER, N=12). Group means ± SEM presented. *$P < 0.05$, vs. WT; †$P < 0.05$, vs. WTX; ‡$P < 0.05$, SUPER vs. TG; §$P < 0.05$ SUPER vs. TGX.

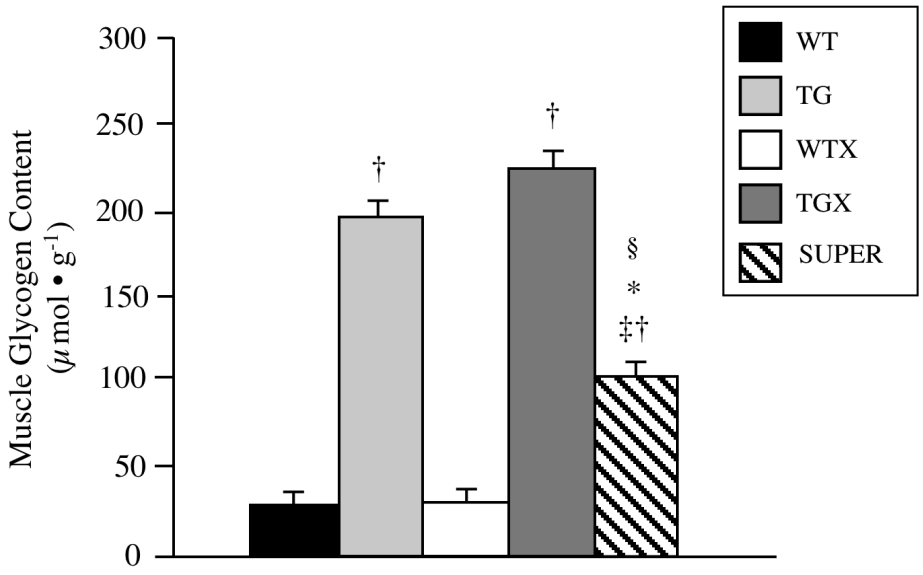


Figure 3.4. Muscle glycogen synthase activity following hindlimb perfusion with either (A) low (0.2 mU • ml^{-1}) or (B) high (10 mU • ml^{-1}) insulin. Groups are wildtype (WT, N=4), transgenic (TG, N=4), exercised wildtype (WTX, N=4), exercised transgenic (TGX, N=5), and wildtype exercised/supercompensated (SUPER, N=5). Group means ± SEM presented. †P < 0.05, TGX vs. WT, WTX, and SUPER; *P < 0.05, TG vs. WT, WTX, and SUPER.

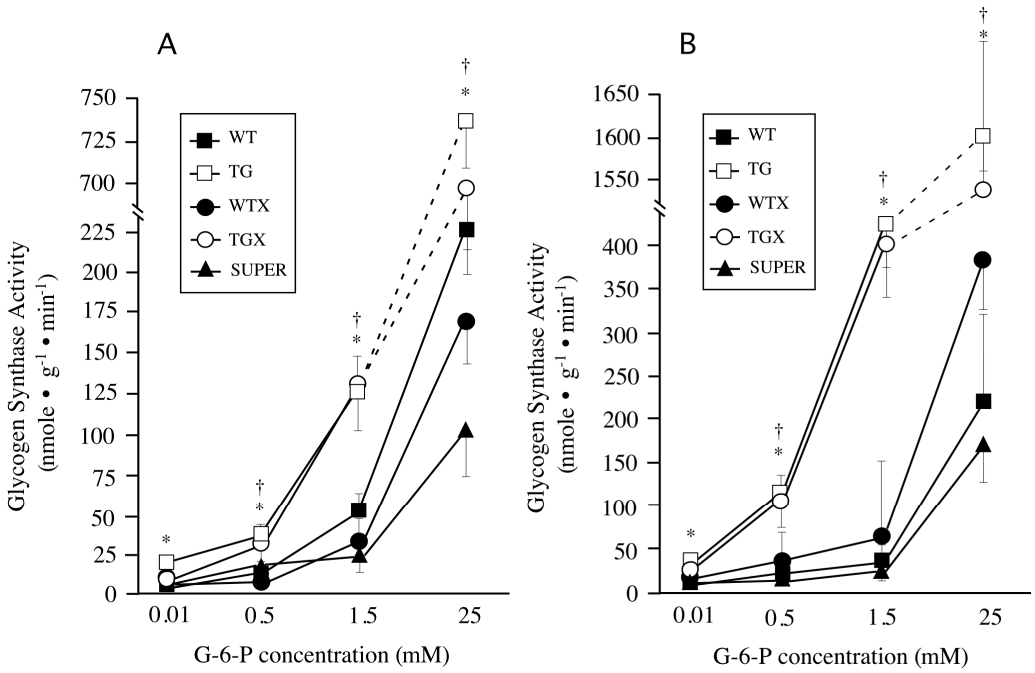


Figure 3.5. Mixed gastrocnemius muscle 2-deoxyglucose uptake following hindlimb perfusion procedure at two insulin concentrations (0.2 mU • ml^{-1} and 10 mU • ml^{-1}). Groups are wildtype (WT, N=6-7), transgenic (TG, N=7), exercised wildtype (WTX, N=6-7), exercised transgenic (TGX, N=6-9), and wildtype exercised/supercompensated (SUPER, N=6). Group means ± SEM presented. †$P < 0.05$, vs. TG within respective insulin concentration; ‡$P < 0.05$ vs. SUPER within respective insulin concentration; *$P < 0.05$, vs. WT within respective insulin concentration.

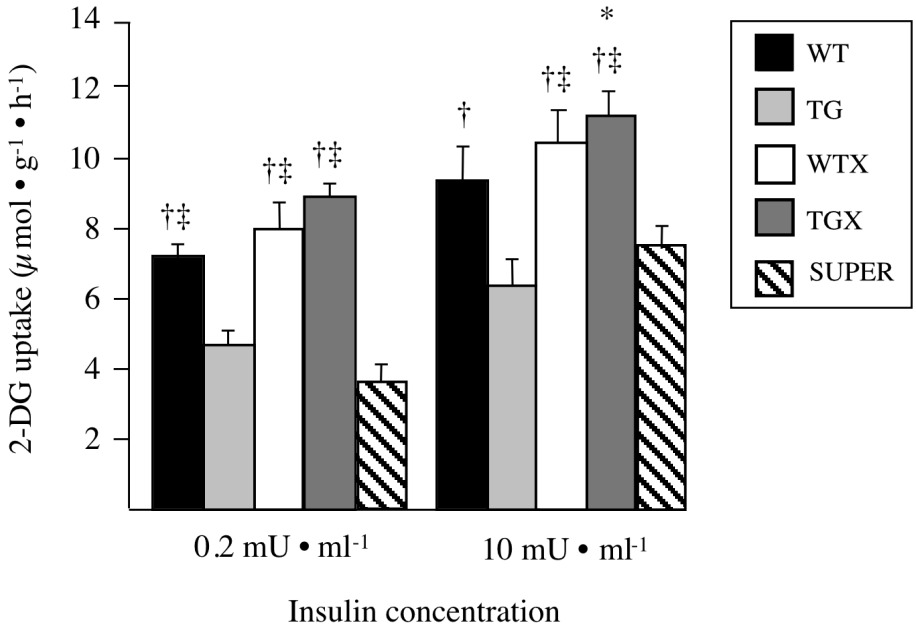


Figure 3.6. GLUT4 protein content in mixed gastrocnemius muscle. Values are expressed as percent of an insulin-stimulated rat heart standard run on all gels. Groups are wildtype (WT, N=10), transgenic (TG, N=12), exercised wildtype (WTX, N=10), exercised transgenic (TGX, N=10), and wildtype exercised/supercompensated (SUPER, N=7). Group means ± SEM presented. †$P < 0.05$, vs. WT.

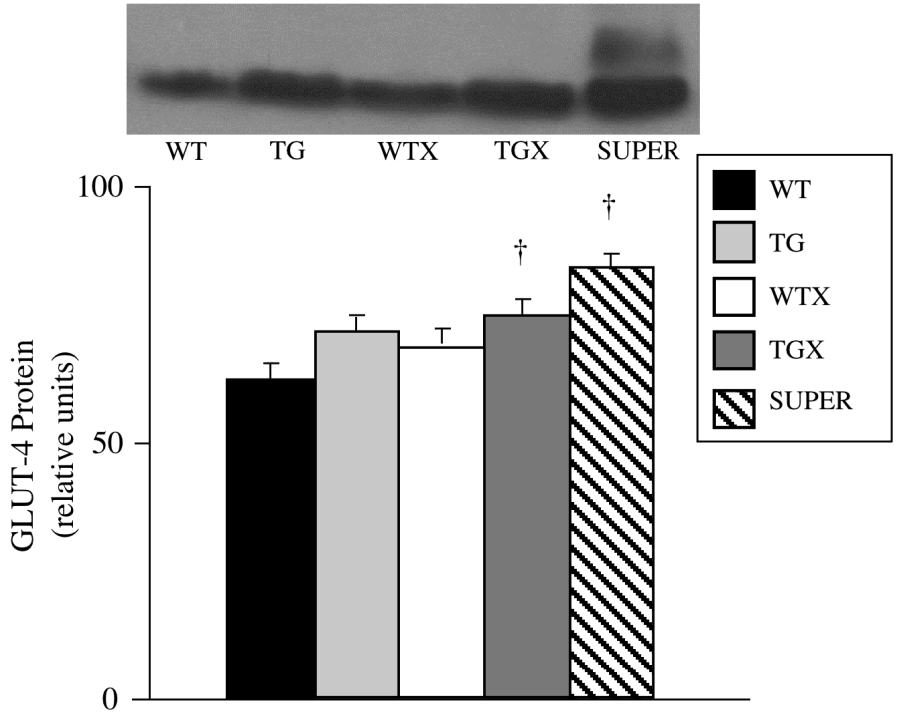


Figure 3.7. Hexokinase activity in mixed gastrocnemius muscle. Values are expressed as µmol • g^{-1} • min^{-1}. Groups are wildtype (WT, N=10), transgenic (TG, N=12), exercised wildtype (WTX, N=10), exercised transgenic (TGX, N=10), and wildtype exercised/supercompensated (SUPER, N=7). Group means ± SEM presented. †$P < 0.05$, vs. WTX; #$P < 0.05$, vs. SUPER.

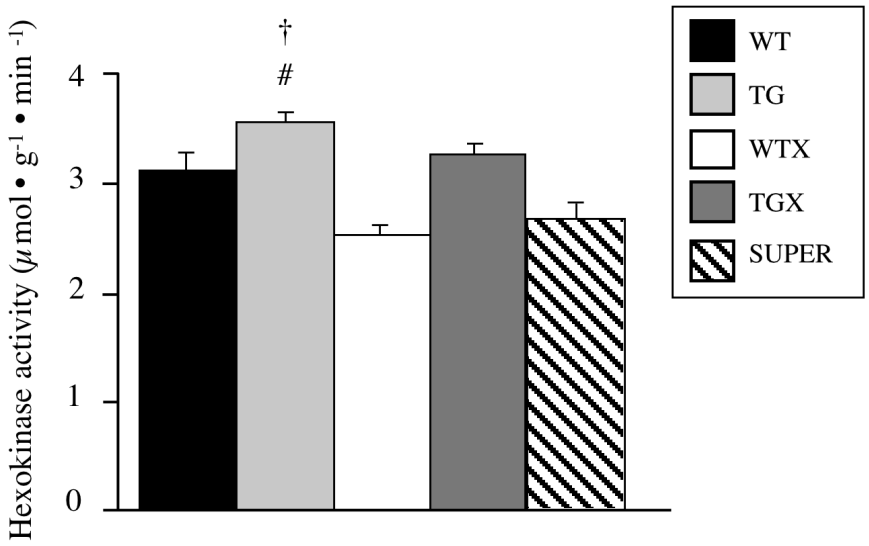


Figure 3.8. Mixed gastrocnemius IRS-1-associated PI3-kinase activity assessed following hindlimb perfusion. Values are expressed as percent of insulin-stimulated mouse standard. Groups are wildtype (WT, N=5), transgenic (TG, N=5-6), exercised wildtype (WTX, N=5), exercised transgenic (TGX, N=6), and wildtype exercised/supercompensated (SUPER, N=5). Group means ± SEM presented. †P < 0.05, vs. WT within respective insulin concentration; ‡*P* < 0.05, vs. SUPER.

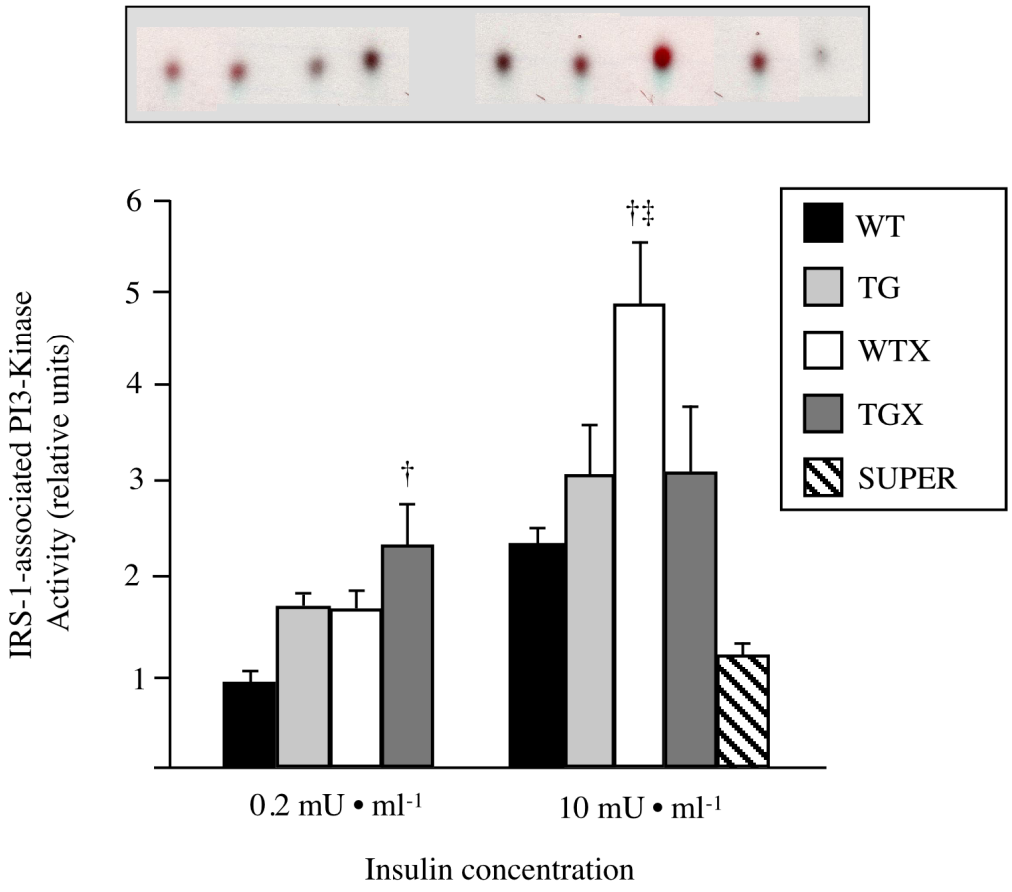


Figure 3.9. Mixed gastrocnemius total PKB/Akt protein as assessed following hindlimb perfusion. Values are expressed as percent of insulin-stimulated mouse standard run on all gels. Groups are wildtype (WT, N=6), transgenic (TG, N=7-8), exercised wildtype (WTX, N=8), exercised transgenic (TGX, N=7-9), and wildtype exercised/supercompensated (SUPER, N=5-6). Group means ± SEM presented. ‡$P < 0.05$, vs. WT. †$P < 0.05$ vs. WTX. *$P < 0.05$ vs. TGX.

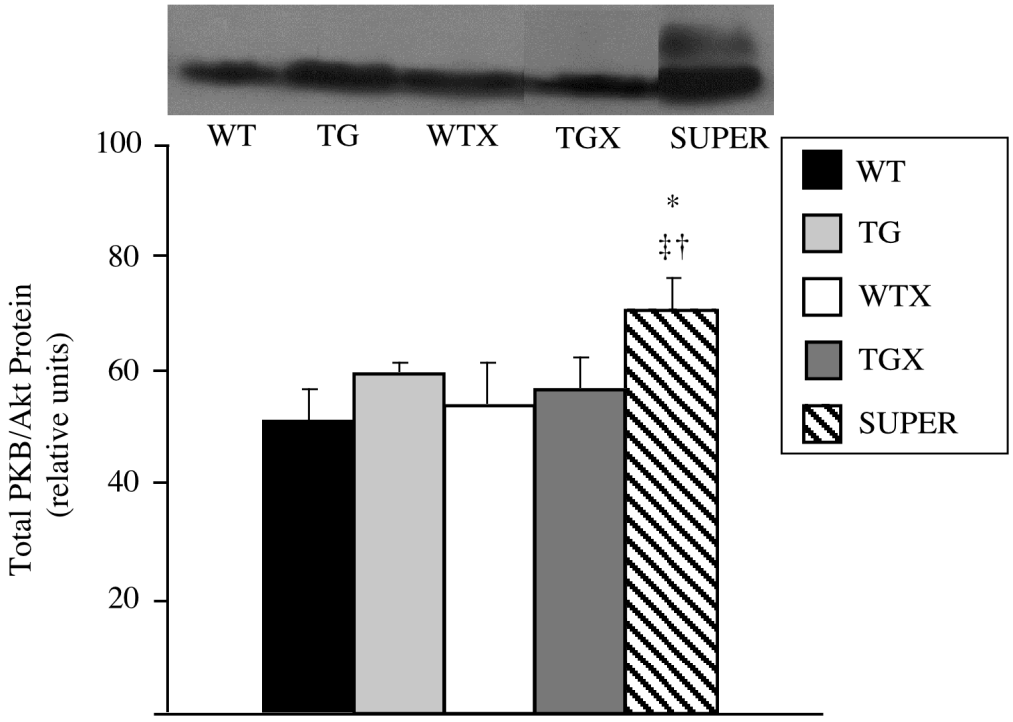
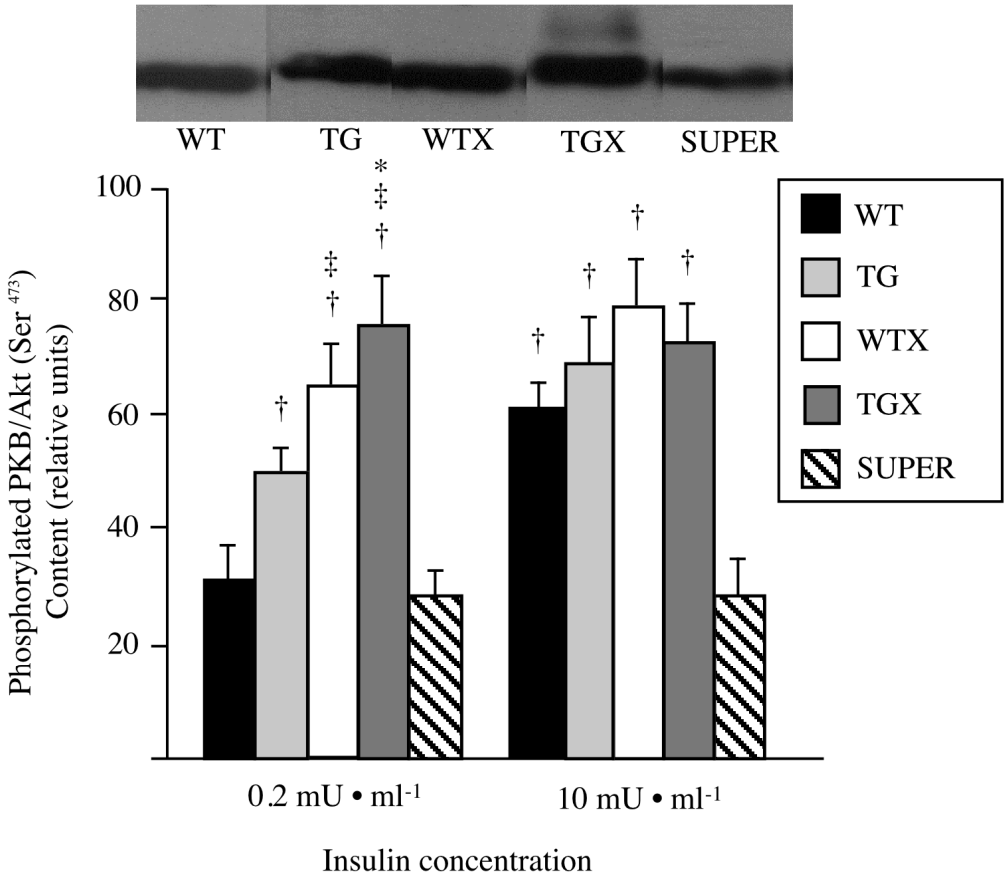


Figure 3.10. Mixed gastrocnemius phosphorylated (Ser^{473}) PKB/Akt as assessed following hindlimb perfusion. Values are expressed as percent of insulin-stimulated mouse standard run on all gels. Groups are wildtype (WT, N=6), transgenic (TG, N=7-8), exercised wildtype (WTX, N=8), exercised transgenic (TGX, N=7-9), and wildtype exercised/supercompensated (SUPER, N=5-6). Group means ± SEM presented. ‡$P < 0.05$, vs. WT within respective insulin concentration; *$P < 0.05$, vs. TG within respective insulin concentration; †$P < 0.05$, vs. SUPER within respective insulin concentration.



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CHAPTER IV: STUDY 2

EFFECTS OF GLYCOGEN SYNTHASE OVEREXPRESSION ON THE INSULIN-INDEPENDENT PHASE OF POST-EXERCISE GLYCOGEN RESYNTHESIS

Abstract

We have determined the effects of glycogen synthase overexpression on muscle glucose metabolism following a lowering of muscle glycogen levels by *in situ* electrical stimulation of the sciatic nerve. Right gastrocnemii from GSL3 transgenic and wildtype mice were subjected to 30 min of stimulation followed by hindlimb perfusion of both hindlimbs. Thirty minutes of contraction significantly reduced muscle glycogen concentration in wildtype (49%) and transgenic (27%) mice although transgenic mice retained 168.8 ± 20.5 μmol • g^{-1} glycogen as compared to 17.7 ± 2.57 μmol • g^{-1} glycogen for wildtype mice. Muscle of transgenic and wildtype mice demonstrated similar pre- (3.6 ± 0.28 μmol • g^{-1} • h^{-1} and 3.9 ± 0.59 μmol • g^{-1} • h^{-1}, for transgenic and wildtype, respectively) and post-contraction (7.9 ± 0.34 μmol • g^{-1} • h^{-1} and 7.0 ± 0.38 μmol • g^{-1} • h^{-1}, for transgenic and wildtype, respectively) insulin-stimulated glucose uptakes. However, the percentage of [^{14}C] glucose incorporated into glycogen was greater in non-contracted (151%) and contracted transgenic muscle (157%) versus muscle of wildtype mice. Also, insulin-stimulated glycogen synthase responsiveness and G-6-P sensitivity, as well as PKB/Akt Ser^{473} phosphorylation were greater in transgenic mice as compared to wildtype mice post-contraction. Contractions increased insulin-stimulated PKB/Akt phosphorylation in muscle of wildtype but not in muscle of transgenic mice. These results indicate that the normal inverse relationship between muscle glycogen concentration and insulin-stimulated glucose uptake is absent in GSL3 transgenic mice. Furthermore, they suggest that

a constitutively active glycogen synthase prevented an elevated glycogen concentration from compromising post-contraction insulin-stimulated muscle glucose uptake and glycogen synthesis.

Introduction

Insulin-stimulated glucose transport during post-exercise recovery appears to be inversely related to the muscle glycogen content (Cartee and Holloszy, 1990; Cartee et al., 1989; Fell et al., 1982; Hespel and Richter, 1990; Richter et al., 1984). In this regard, it has been suggested that glycogen content may influence the number of glucose transporters that can be actively associated with the plasma membrane. Recent studies utilizing a cell surface GLUT4 protein labeling technique have demonstrated an inverse relationship between muscle glycogen content and GLUT4 transporters associated with the plasma membrane following insulin stimulation (Derave et al., 2000; Etgen et al., 1996; Kawanaka et al., 1999). Increased muscle glycogen content is also associated with decreased activity of PKB/Akt (Derave et al., 2000; Kawanaka et al., 2000), a key insulin signaling protein involved in stimulation of GLUT4 protein translocation (Cheatham and Kahn, 1995; Hajduch et al., 1998; Kohn et al., 1996; Tanti et al., 1997). In addition, an inverse relationship between glycogen concentration and the activity of glycogen synthase, the rate-limiting enzyme for glycogen synthesis, also exists (Danforth, 1965; Laurent et al., 2000).

Evidence from recent research utilizing the GSL3 transgenic mouse model challenges the normal inverse relationship between muscle glycogen levels and insulin-stimulated glucose transport. GSL3 transgenic mice overexpress a constitutively active form of glycogen synthase (10-fold) which is associated with a markedly elevated (5-fold) muscle glycogen concentration (Manchester et al.,

1996). Using this model, Azpiazu et al. (2000) demonstrated that overexpression of glycogen synthase is sufficient to promote glycogen accumulation without reducing insulin action on muscle glucose uptake. Since GSL3 transgenic muscle did not demonstrate the classic insulin resistance typically associated with an elevated muscle glycogen level, this raises the question of whether or not insulin-stimulated glucose uptake is influenced by muscle glycogen. We have previously assessed *in situ* insulin-stimulated muscle glucose uptake in GSL3 transgenic muscle following exhaustive running exercise combined with carbohydrate restriction (Unpublished data). However, we were unable to lower muscle glycogen concentrations of GSL3 mice using this experimental protocol. Thus, we could not investigate the effects of glycogen on insulin-stimulated muscle glucose uptake post-exercise. In an effort to assess the influence of muscle glycogen on insulin action following muscular contractions, we electrically stimulated muscle of wildtype and GSL3 transgenic mice *in situ* via the sciatic nerve. Our results suggest that insulin action immediately following muscular contraction is improved independent of muscle glycogen concentration.

Methods

Animals and Experimental Design. GSL3 transgenic and wildtype littermates [(C57BL6 X CBA)F_1] weighing 20-35 g (n=25) were used in these experiments. The GSL3 transgenic mice overexpress a constitutively active form of glycogen synthase [GS(2,3a)], a rabbit skeletal muscle glycogen synthase having Ser to Ala mutations at sites 2 and 3a (donated by Dr. John Lawrence,

University of Virginia, Charlottesville, VA). The generation of the transgenic line is described by Manchester et al. (1996). Transgenic expression of the constitutively active glycogen synthase is 10 times higher compared to wildtype muscle, with 5 times greater expression in fast twitch versus slow twitch muscle (Manchester et al., 1996). Upon receiving the mice, they were individually housed at The University of Texas Animal Resource Center on a 12:12 h light-dark cycle. Laboratory chow and water were provided ad libitum and the animal room temperature was maintained at 21ºC. Animals were transferred to the laboratory at least 24 h prior to the first experimental procedures following the same light cycle. The University of Texas Animal Care and Use Committee approved all procedures for this study.

Muscle Contraction Procedure. Following a 6 h fast, GSL3 transgenic (n=13) and wildtype (n=12) mice were anesthetized with pentobarbitol sodium (6.5 mg • 100 g^{-1} body weight). The skin from the right hindlimb was reflected and a section of the calcaneous, with the Achilles tendon still attached, clipped from the foot. The triceps surae muscle group was retracted from the tibia and the distal tendon (detached from the bony insertion) of the muscle group was attached to an isometric force transducer. However, the distal insertions of the plantaris and soleus muscles were sectioned and teased away from the gastrocnemius so, upon stimulation of the sciatic nerve, only the gastrocnemius was producing an isometric contraction. The sciatic nerve was isolated and placed in-line, via hook electrode, with a Grass Stimulator (Grass Instruments, Quincy, MA). Both the non-stimulated left hindlimb and the mouse torso were immobilized in a specially

designed cradle and gastrocnemius length adjusted to achieve peak twitch tension (Pt). Pt and peak tetanic tension (Po) were assessed (Series 300 B Lever System, Aurora Scientific Inc., Ontario, Canada) with supramaximal stimulation strength at the optimal length for the muscle contracting at 90º to the knee joint.

The goal of this stimulation procedure was to reduce muscle glycogen prior to the assessment of 2-deoxyglucose uptake. Therefore, a modified version of the protocol described by Johannsson et al. (1996a & 1996b) and Roy et al. (1997) was employed. The gastrocnemius was stimulated for 30 min using supramaximal (8 V) trains composed of 1 ms square-wave pulses firing at 100 Hz. Trains of 200 ms were delivered at a rate of 30 per min (1 per 2 sec). Tetanic tension was assessed throughout the stimulation period. Gastrocnemius muscle cross sectional area was estimated by dividing wet weight by the measured optimal muscle length (cm) multiplied by the fiber to muscle length ratio for rodent gastrocnemius (0.4) multiplied by mammalian muscle specific gravity (1.06 g/cm^3). Specific force (N/cm^2) was then estimated by dividing muscle Po, expressed in newtons (N), by the estimated cross-sectional area (cm^2) (Brooks and Faulkner, 1988). Following the stimulation period, the mice were prepared for *in situ* hindlimb perfusion to measure insulin-stimulated [^{3}H] 2-deoxyglucose uptake or to determine [^{14}C] glucose incorporation into glycogen using a submaximal insulin concentration (0.2 mU • ml^{-1}).

Hindlimb perfusion. The perfusion surgical technique was similar to that described previously (Brozinick et al., 1996; Ivy et al., 1983). Major vessels branching from the abdominal aorta and vena cava except the common iliac artery

and iliac vein were ligated. Heparin (100 U) was injected into the inferior vena cava just inferior to the diaphragm. Following the heparin injection, the descending aorta and inferior vena cava were cannulated as previously described (Ivy et al., 1983). Catheters were placed in line with the non-circulating hindlimb perfusion apparatus providing a 37ºC, continuously gassed (95% O_2/5% CO_2) perfusate flow of 1.8 ml • min^{-1}. Both hindlimbs were perfused during the equilibrium (10 min) and tracer (20 min) periods. Perfusates consisted of 6% bovine serum albumin (BSA) in Krebs-Henseleit buffer (KHB, pH 7.4) with 0.2 mM pyruvate. Tracer perfusion periods varied between assessment of glucose uptake and glucose incorporation and are described below. Immediately following the perfusion period, the gastrocnemius from both hindlimbs were excised, frozen with Wollenberg tongs cooled in liquid N_2 and stored frozen (-80ºC) until further analysis.

Determination of 2-Deoxyglucose Uptake. During the perfusion tracer period, the perfusate contained 6 mM [1,2-^{3}H] 2-deoxyglucose (7.5 µCi • $mmol^{-1}$; ICN Biochemicals, Costa Mesa, CA), 2 mM [U-^{14}C] mannitol (60 µCi • $mmol^{-1}$; ICN Biochemicals) and 0.2 mU • ml^{-1} insulin (Humulin R-100, Eli Lilly, Indianapolis, IN). Freeze-clamped gastrocnemius muscles from the perfused wildtype (n = 6) and transgenic (n = 6) hindlimbs were sectioned and weighed frozen. A 60-100 mg piece of mixed-fiber muscle was dissolved in 1 ml 1 N KOH by incubating for 15 min at 65ºC, mixed, and incubated an additional 5 min at 65ºC. An equal volume of 1 N HCl was added to the digested samples, mixed, and aliquots of the neutralized samples counted for [^{3}H] and [^{14}C] DPM (Beckman

LS 6000SC, Fullerton, CA). Muscle 2-deoxyglucose uptake was calculated from the specific activity of the original perfusate after subtracting out the appropriate volume for extracellular space as determined from radiolabelled mannitol in the muscle sample. The mixed gastrocnemius was used because it exhibits a high transgene expression but contains similar GLUT4 content in wildtype and GSL3 transgenic mice (Manchester et al., 1996).

[^{14}C] Glucose incorporation into glycogen. [^{14}C] Glucose incorporation into glycogen was assessed in a different group of wildtype (n = 7) and transgenic (n = 5) hindlimbs as previously described (Willems et al., 1991). The perfusion surgery and preparation was essentially the same as described for 2-deoxyglucose uptake during the pre-tracer perfusion period. During the 20 min tracer perfusion period both hindlimbs were perfused with 6 mM glucose (0.15 µCi • ml^{-1} D-[^{14}C(U)] glucose) (ICN Biochemicals) with a submaximal insulin concentration (0.2 mU • ml^{-1}). Following the perfusion period, both gastrocnemii were excised, weighed frozen and placed in screw-top test tubes containing 1 ml of 30% KOH saturated with Na_2SO_4. Samples were digested by incubating the tubes for 30 min at 100ºC. After the incubation, the tubes were cooled to room temperature and the glycogen precipitated overnight at 4ºC after the addition of 1:2 volumes of 95% ethanol. The glycogen was pelleted by centrifugation and resuspended in distilled H_2O. An aliquot of this resuspension was used for determination of muscle glycogen concentration as per Lo et al. (1970). A second aliquot was transferred to a scintillation vial containing 5 ml of scintillation fluid and the rate of [^{14}C] glucose incorporation into glycogen determined.

Measurement of glycogen synthase activity. Glycogen synthase was measured by direct incorporation of [U-^{14}C] UDP-1-glucose (ICN Biochemicals) into glycogen with several concentrations of G-6-P (0.01, 0.5, 1.5, and 25 mM) as previously described (Sherman et al., 1988). Glycogen synthase total activity was determined in the presence of 25 mM G-6-P. The fractional velocities of glycogen synthase were determined by dividing the enzyme activity of 0.5 and 1.5 mM G-6-P by that of maximal (25 mM) G-6-P glycogen synthase activity and are expressed as percentages. In addition, the G-6-P +/- activity ratio was calculated by dividing the glycogen synthase activity measured in the presence of 0.01 mM G-6-P divided by the total activity.

*Total and Phosphorylated PKB/Akt-1*α. Three isoforms of PKB/Akt have been identified. The primary insulin-stimulated isoform in rodent muscle is PKB/Akt-1α (Turinsky and Damrau-Abney, 1999; Walker et al., 1998). In addition, post-exercise insulin-stimulated PKB/Akt-1α activity was inversely related to skeletal muscle glycogen content in the study by Derave et al. (2000). Therefore, PKB/Akt-1α is was the isoform assessed in the present study. Frozen muscle samples were prepared as described by Kawanaka et al. (2000). Muscle pieces (30 mg) were homogenized in ice-cold buffer containing 50 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffer, 1 mM EDTA, and 250 mM sucrose, pH 7.4), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1.0 mM EDTA, 10 mM $Na_4P_2O_7$, 100 mM NaF, 2.0 mM Na_3VO_4, aprotinin (10 μg • ml^{-1}), leupeptin (10 μg • ml^{-1}), pepstatin (0.5 μg • ml^{-1}), 1% Igepal and 2 mM phenylmethylsulfonyl fluoride. For quantification of

phosphorylated PKB/Akt-1α, aliquots of the 200,000 *g* supernatant were treated with 2x Laemmli sample buffer containing 100 mM dithiothreitol and boiled for 5 min. Samples (80 μg protein) were subjected to SDS-PAGE (10% resolving gel) and transferred to nitrocellulose membranes. An insulin-stimulated mouse gastrocnemius muscle standard (80 μg) was run on each gel. The membranes were blocked in 5% nonfat dry milk in TBS containing 0.1% Tween 10 (pH 7.5) for 1 h. Following a rinse in 0.1% Tween 10, the membranes were incubated with sheep antiphospho-PKB/Akt-1α (Ser^{473}) antibody (Upstate Biotechnology Inc.) for 4 h. Membranes were rinsed in 0.1% Tween 10 and incubated with horseradish peroxidase-conjugated rabbit anti-sheep IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) for 60 min. Antibody-bound protein was visualized using an ECL Western blot detection kit (Amersham) according to the manufacturer's instructions. Phosphorylated PKB/Akt of samples was expressed relative to the insulin-stimulated mouse standard.

Total PKB/Akt-1α protein content was assessed on the nitrocellulose membranes used for phosphorylated PKB/Akt-1α. Membranes were stripped of antibodies by incubation with stripping buffer containing 100 mM 2-mercaptoethanol, 2% sodium dodecyl sulfate, 62.5 mM Tris HCl (pH 6.7) for 30 min. Membranes were then washed three times for 15 min in TTBS buffer. Membranes were blocked overnight, washed three more times for 15 min in TTBS, and incubated with sheep anti-Akt/PKB-1α (1:1000 vol/vol) (Upstate Biotechnology Inc.) for 4 h with gentle agitation. Following three more TTBS

washes, antibody-bound protein was visualized using ECL (Amersham) and total PKB/Akt-1α was expressed relative to the insulin-stimulated mouse standard.

Statistics. One-way analysis of variance was performed on *in situ* electrical stimulation performance data. Two-way analysis of variance was performed on all tissue analyses. Post hoc tests were performed using Fishers's Protected LSD with differences considered statistically significant if $P \leq 0.05$.

Results

Muscle contractile performance. Gastrocnemius Pt, optimal muscle length, muscle wet weight and estimated muscle cross-sectional area were not significantly different between wildtype and GSL3 transgenic mice (Table 4.1). Transgenic muscle Po was 14% lower than wildtype (Table 4.1). This difference was significant at the $P < 0.06$ level. Specific force was significantly lower in GSL3 transgenic muscle (16.1 ± 0.88 N/cm^2) compared to wildtype muscle (21.4 ± 1.5 N/cm^2) (Table 4.1). Gastrocnemius tetanic tension production declined to 48% of Po for wildtype mice and 61% of Po in GSL3 transgenic mice by 5 min of stimulation (Figure 4.1). Tetanic tension production remained essentially unchanged from 5 min throughout the 30 min stimulation period. Wildtype muscle contractile performance was similar to those found by Johannson et al. (1996a), using a similar stimulation protocol in the rat.

Muscle glycogen concentration. There was a significant genotype and treatment effect with respect to muscle glycogen concentration. Glycogen concentration of gastrocnemius from transgenic mice was approximately 7-fold

greater than that of wildtype mice (Figure 4.2). Muscle contraction significantly lowered muscle glycogen in both genotypes. The reduction in muscle glycogen was 49% in wildtype gastrocnemius and 27% in transgenic gastrocnemius. However, in absolute terms, 30 min of contraction reduced glycogen by 63 μmol • g^{-1} wet weight in transgenic muscle and 17 μmol • g^{-1} wet weight in wildtype muscle. Quadriceps muscle glycogen content was unchanged by stimulation in either genotype (data not shown) demonstrating that the decline in gastrocnemius muscle glycogen was specific to the muscle stimulated by the sciatic nerve.

Glycogen synthase. A significant genotype effect was evident for glycogen synthase fractional velocities at 1.5 and 0.5 mM G-6-P, as well as -/+ G-6-P activity ratio. These measures were markedly lower in GSL3 transgenic muscle compared to wildtype and unchanged by treatment in either genotype (Table 4.2). However the total activity of glycogen synthase (25 mM G-6-P), expressed in nmol • g^{-1} • min^{-1}, was significantly greater in non-contracted (1197%) and contracted (1761%) transgenic compared to muscle from the respective wildtype treatment. In addition, allosteric activation of glycogen synthase was greater at G-6-P concentrations of 1.5 (361%) and 0.5 mM (142%) in non-contracted transgenic muscle compared to muscle from wildtype mice. Following 30 min of contractions, glycogen synthase allosteric activation was greater (1062% and 291% for 1.5 and 0.5 mM, respectively) in transgenic muscle compared to wildtype muscle. Contraction of muscle of transgenic mice resulted in greater total glycogen synthase activity (110%) and allosteric activation of glycogen synthase at G-6-P concentrations of 1.5 (131%) and 0.5 mM (62%) compared to

non-contracted transgenic muscle. No differences were observed between any group during exposure to 0.01 mM G-6-P.

2-Deoxyglucose uptake. Genotype had no effect on insulin-stimulated glucose uptake of the gastrocnemius muscle. There was, however, a significant treatment effect as 30 min of contraction resulted in a 2-fold increase in insulin-stimulated glucose uptake (Figure 4.4). This 2-fold increase occurred in both transgenic and wildtype muscle.

[^{14}C] Glucose incorporation into glycogen. Significant genotype and treatment effects were observed with respect to insulin-stimulated glucose incorporation into glycogen. Glucose incorporation into glycogen in transgenic muscle was significantly higher prior to (151%) and following (157%) contraction than in the corresponding wildtype muscle (Figure 4.5). However, contraction resulted in a 1.9-fold increase in insulin-stimulated incorporation of glucose in muscle of both genotypes.

Total and Phosphorylated PKB/Akt. Total PKB/Akt protein was similar in wildtype and GSL3 transgenic muscle and was not altered by 30 min of contraction (Figure 4.6). Significant genotype, treatment, and interaction effects were evident with respect to insulin-stimulated PKB/Akt Ser^{473} phosphorylation. Insulin-stimulated PKB/Akt Ser^{473} phosphorylation was significantly higher in muscle of transgenic mice as compared to muscle of wildtype mice (Figure 4.7). This difference was significant prior to (212%) and following (52%) muscle contraction. Contraction significantly increased insulin-stimulated PKB/Akt phosphorylation, but only in wildtype muscle (131%).

Discussion

Insulin-stimulated glucose uptake during post-exercise recovery appears to be inversely related to the muscle glycogen concentration (Cartee and Holloszy, 1990; Cartee et al., 1989; Fell et al., 1982; Hespel and Richter, 1990; Richter et al., 1984). However, evidence from recent research utilizing the GSL3 transgenic mouse model challenges this relationship (Azpiazu et al., 2000) In an effort to assess the influence of muscle glycogen on insulin action following muscular contractions, we electrically stimulated muscle of GSL3 transgenic and wildtype mice *in situ* via the sciatic nerve. Our results suggest that insulin action immediately following muscular contraction is improved independent of muscle glycogen concentration.

Insulin-stimulated muscle glucose uptake was similar in transgenic and wildtype mice prior to and following muscle contraction. However, this occurred in the face of a markedly greater transgenic muscle glycogen concentration. These findings suggest that the normal inverse relationship between elevated muscle glycogen levels and insulin action on glucose uptake is absent in GSL3 transgenic mice.

When muscle glycogen is elevated above normal levels, the ability of insulin to activate glucose transport (Cartee et al., 1989; Derave et al., 2000; Fell et al., 1982; Jensen et al., 1997; Kawanaka et al., 2000; Richter et al., 1984) is significantly depressed. Thus, it has been suggested that glycogen may have some control over the number of glucose transporters that can be actively associated with the plasma membrane. Recent studies utilizing the ATB-BMPA cell surface

GLUT4 labeling technique have demonstrated an inverse relationship between muscle glycogen content and GLUT4 protein associated with the plasma membrane following insulin stimulation (Derave et al., 2000; Etgen et al., 1996; Kawanaka et al., 1999). The inhibition of GLUT4 translocation by elevated muscle glycogen might occur directly via the glycogen macromolecule itself or indirectly through glycogen-mediated inhibition of PKB/Akt (Derave et al., 2000; Kawanaka et al., 2000). PKB/Akt is a key insulin signaling protein involved in stimulation of GLUT4 translocation (Cheatham and Kahn, 1995; Hajduch et al., 1998; Kohn et al., 1996; Tanti et al., 1997). An inverse relationship between glycogen concentration and the activity of glycogen synthase, the rate-limiting enzyme for glycogen synthesis (Danforth, 1965; Laurent et al., 2000) also exists.

In addition to a putative role in GLUT4 translocation to the cell surface, PKB/Akt may catalyze the phosphorylation and inactivation of GSK-3 in skeletal muscle (Cross et al., 1995). GSK-3, in turn, has been implicated in inhibition of glycogen synthase (Cross et al., 1997; Ueki et al., 1998) as well as feedback inhibition of IRS-1 facilitated PI3-kinase activity (Eldar-Finkelman and Krebs, 1997; Summers et al., 1999). Thus, PKB/Akt may play a pivotal role in both insulin regulation of glucose transport and glycogen synthase activity.

Insulin-stimulated glucose uptake was normal while PKB/Akt phosphorylation was significantly higher in transgenic muscle compared to wildtype. These findings are in stark contrast to the inverse relationship normally observed between muscle glycogen concentration and insulin-stimulated glucose uptake and PKB/Akt phosphorylation. Thus, the normal suppression of insulin

signaling and glucose uptake associated with elevated muscle glycogen concentrations is disrupted in transgenic muscle.

Why resting and post-exercise insulin-stimulated PKB/Akt phosphorylation remains highly active in GSL3 transgenic muscle despite elevated muscle glycogen concentrations (>200 μmol • g^{-1}) remains unclear. Derave et al. (2000) and Kawanaka et al. (2000) both demonstrated that elevated muscle glycogen concentration (~60-130 μmol • g^{-1}) in rats was associated with an attenuation of insulin-stimulated increases in PKB/Akt phosphorylation. Furthermore, we have previously demonstrated this attenuation in wildtype mice with muscle glycogen concentrations of 103 μmol • g^{-1} (Unpublished data).

The maintenance of PKB/Akt activity in the GSL3 transgenic muscle despite above normal glycogen concentrations could facilitate GLUT4 translocation to the cell surface as well as inhibition of GSK-3. Inhibition of GSK-3, in turn, might alleviate feedback inhibition of IRS-mediated insulin signaling. Thus, activation PKB/Akt could enhance insulin-stimulated muscle glucose uptake by two potential mechanisms in GSL3 transgenic muscle. These results may also be related to the constitutively active glycogen synthase, which is over expressed in GSL3 transgenic muscle. The influence of glycogen on PKB/Akt activity may be dependent upon the ability of glycogen to depress glycogen synthase activity. This might represent a putative, yet unknown, association between the activation states of glycogen synthase and PKB/Akt, both of which are normally inhibited when muscle glycogen has been elevated above normal.

Our observations that insulin-stimulated muscle glucose uptake was similar yet [^{14}C] glucose incorporation into glycogen was greater in resting transgenic muscle compared to wildtype correspond with those observed by Azpiazu et al. (2000). Taken together, these findings suggest that overexpression of glycogen synthase may facilitate a repartitioning of intracellular glucose intermediates towards glycogen synthesis as opposed to the glycolytic, hexosamine, and oxidative pathways. This repartitioning may prevent accumulation of G-6-P, glucosamine, and citrate, all of which may act to suppress insulin-stimulated glucose uptake. Furthermore, overexpression of glycogen synthase is sufficient to promote glycogen accumulation while preventing muscle glycogen levels from compromising insulin action on muscle glucose uptake.

In the present study, differences in peak tetanic tension and specific force in GSL3 transgenic muscle compared to wildtype muscle could not be explained by muscle wet weight or estimated cross sectional area. Differences in peak tetanic tension and specific force may result, at least in part, from the 7-fold greater glycogen content in the GSL3 transgenic muscle. Considering the water content associated with glycogen storage (Geddes, 1986), the extensive glycogen macromolecule in GSL3 transgenic muscle may have disrupted myofibrillar mechanics.

In summary, we have demonstrated that a constitutively active glycogen synthase prevents glycogen from compromising post-contraction insulin-stimulated muscle glucose uptake and glucose incorporation into glycogen. Overexpression of a constitutively active form of glycogen synthase may facilitate

a repartitioning of intracellular glucose intermediates towards glycogen synthesis as opposed to the glycolytic, hexosamine, and oxidative pathways following contractions. Furthermore, our PKB/Akt phosphorylation data support the possibility that the normal mechanisms responsible for the inverse relationship between muscle glycogen concentration and glucose uptake may be absent in GSL3 transgenic muscle. This may represent a putative, yet unknown, association between the activation states of glycogen synthase and PKB/Akt, both of which are normally inhibited during conditions where muscle glycogen has been elevated.

Table 4.1. Gastrocnemius muscle contractile performance. Peak twitch tension (Pt), peak tetanic tension (Po), optimal muscle length (Lo), wet weight, estimated cross-sectional area (CSA), and specific force are presented.

	Wildtype (N=6)	Transgenic (N=7)
Pt (N)	1.07 ± 0.03	1.11 ± 0.03
Po (N)	3.30 ± 0.10	2.85 ± 0.18*
Lo (cm)	1.68 ± 0.07	1.63 ± 0.09
Wet weight (g)	0.11 ± 0.01	0.12 ± 0.01
CSA (cm^2)	0.16 ± 0.01	0.18 ± 0.02
Specific force (N/cm^2)	21.4 ± 1.5	16.1 ± 0.88†

Values are means ± SEM. (*) $P < 0.06$, vs. WT; (†) $P < 0.05$, vs. WT.

Table 4.2. Gastrocnemius muscle fractional velocity at 1.5 mM and 0.5 mM G-6-P; and -/+ G-6-P activity ratio following hindlimb perfusion with either low (0.2 mU • ml^{-1}) insulin concentration. Fractional velocities are expressed in percentages. Groups are non-stimulated wildtype (N=6), stimulated wildtype (N=5), non-stimulated transgenic (N=5), and non-stimulated transgenic (N=5).

Group	Treatment	Fract. Vel. (1.5 mM G-6-P)	Fract. Vel. (0.5 mM G-6-P)	Activity Ratio (-/+ G-6-P)
WT	non-stimulated	43.1 ± 11.6	35.2 ± 11.1	0.36 ± 0.097
	stimulated	48.0 ± 13.9	36.3 ± 11.1	0.31 ± 0.087
TG	non-stimulated	6.56 ± 1.9 ‡	15.8 ± 3.4 ‡	0.04 ± 0.014 ‡
	stimulated	5.3 ± 0.3 ‡	18.4 ± 1.9 ‡	0.02 ± 0.006 ‡

‡$P < 0.05$, vs. both WT groups.

Figure 4.1. Gastrocnemius tetanic tension produced during 30 min of *in situ* electrical stimulation via the sciatic nerve. Groups are stimulated wildtype (N=5) and stimulated transgenic (N=5). Group means ± SEM presented. †$P < 0.06$, transgenic vs. wildtype.

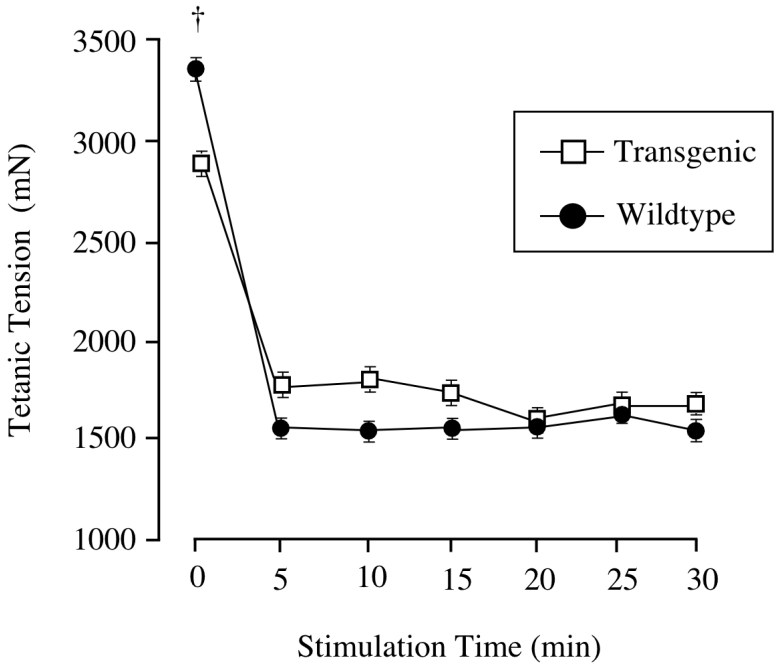


Figure 4.2. Muscle glycogen content in mixed gastrocnemius following 30 min of *in situ* electrical stimulation. Groups are non-stimulated wildtype (N=9) and transgenic (N=9) and stimulated wildtype (N=5) and transgenic (N=5). Group means ± SEM presented. †$P < 0.05$, vs. respective wildtype treatment. *P <0.05, vs. respective non-stimulated (NON) groups.

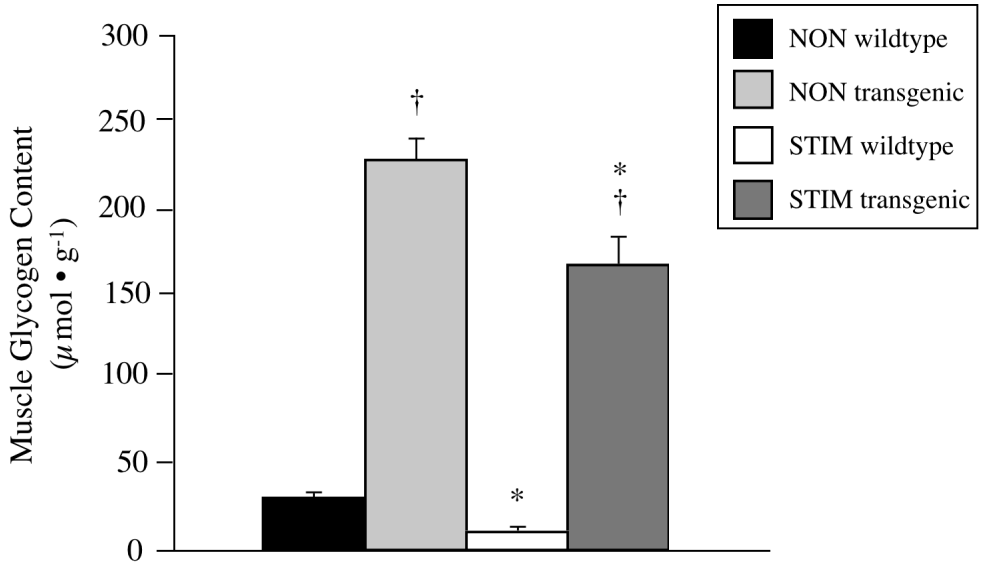


Figure 4.3. Mixed gastrocnemius glycogen synthase activity following hindlimb perfusion (0.2 mU • ml^{-1} insulin) following 30 min *in situ* electric stimulation protocol. Groups are non-stimulated wildtype (N=6) and transgenic (N=5) and stimulated wildtype (N=5) and transgenic (N=5). Group means are presented. †$P < 0.05$, stimulated (STIM) transgenic vs. all groups. ‡$P < 0.05$, non-stimulated (NON) transgenic vs. all groups.

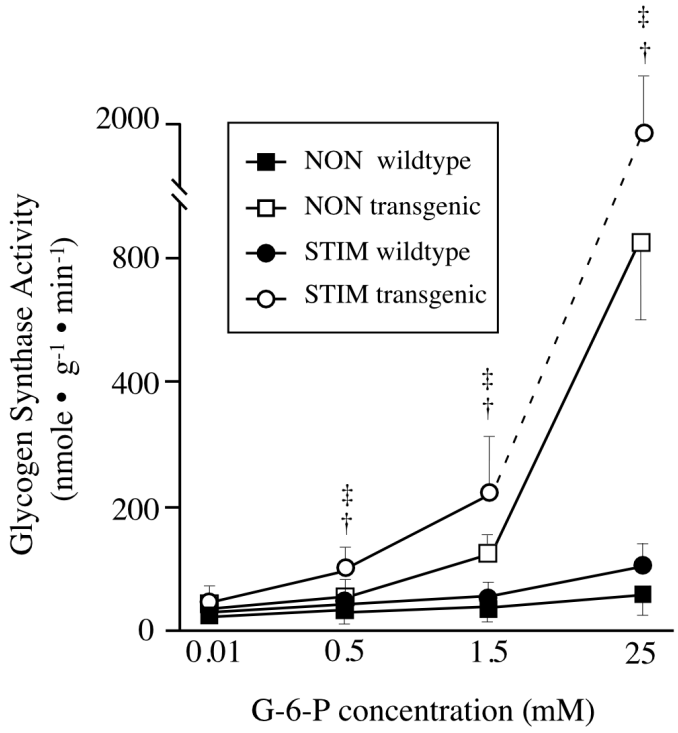


Figure 4.4. Insulin-stimulated 2-deoxyglucose uptake in mixed gastrocnemius muscle following 30 min of *in situ* electrical stimulation. Groups are non-stimulated wildtype (N=6) and transgenic (N=6) and stimulated wildtype (N=6) and transgenic (N=6). Group means ± SEM presented. *$P < 0.05$, vs. respective non-stimulated (NON) groups.

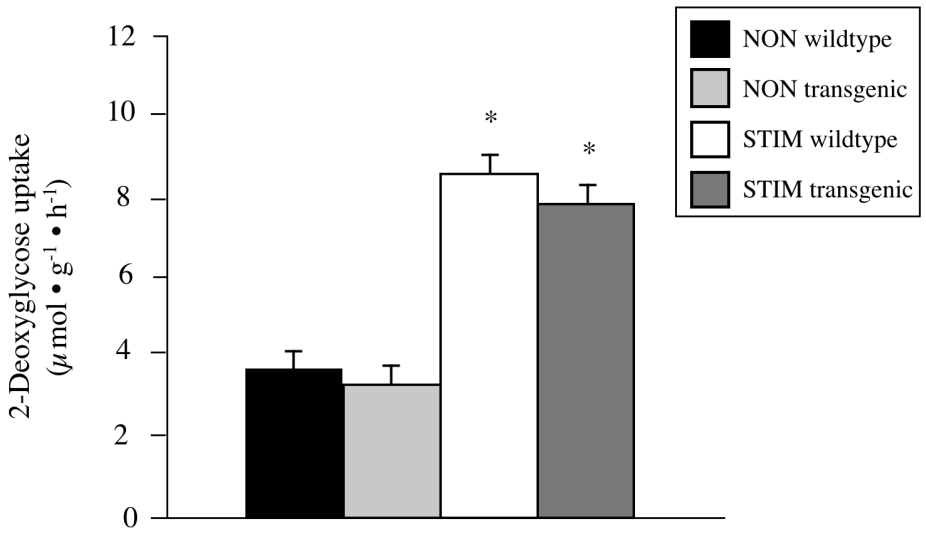


Figure 4.5. Insulin-stimulated [^{14}C] glucose incorporation into glucose from mixed gastrocnemius following 30 min of *in situ* electrical stimulation. Groups are non-stimulated wildtype (N=9) and transgenic (N=9) and stimulated wildtype (N=5) and transgenic (N=5). Group means ± SEM presented. †$P < 0.05$, vs. respective wildtype treatment. *$P < 0.05$, vs. respective non-stimulated (NON) treatment.

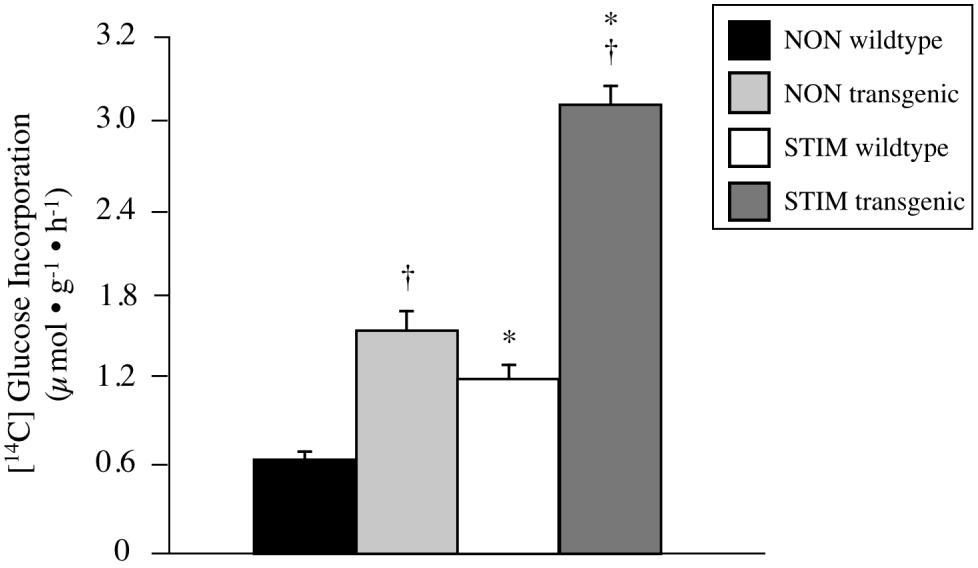


Figure 4.6. Mixed gastrocnemius total PKB/Akt protein as assessed following hindlimb perfusion. Groups are non-stimulated wildtype (N=6) and transgenic (N=6) and stimulated wildtype (N=6) and transgenic (N=6). Values are expressed as percent of insulin-stimulated mouse standard run on all gels. Group means ± SEM presented.

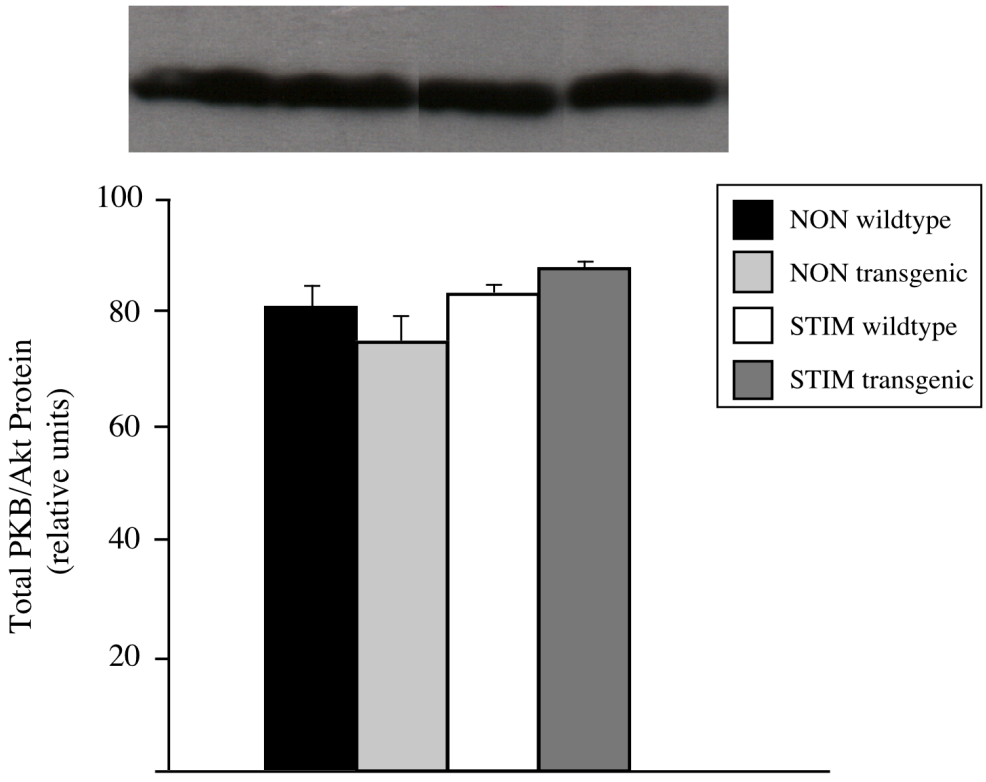
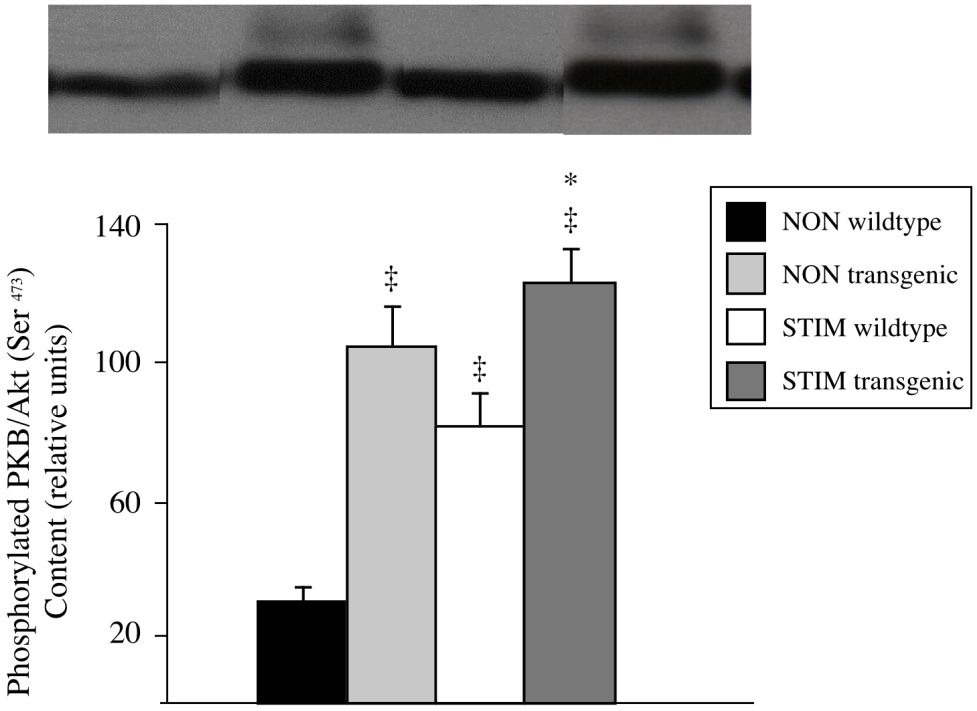


Figure 4.7. Mixed gastrocnemius phosphorylated PKB/Akt (Ser^{473}) as assessed following hindlimb perfusion (0.2 mU • ml^{-1}). Values are expressed as percent of insulin-stimulated mouse standard run on all gels. Groups are non-stimulated wildtype (N=6) and transgenic (N=6) and stimulated wildtype (N=6) and transgenic (N=6). Group means ± SEM presented. ‡*P* < 0.05, vs. non-stimulated (NON) wildtype. **P* < 0.05, vs. stimulated (STIM) wildtype.



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CHAPTER V: EXTENDED REVIEW OF THE LITERATURE

Introduction

Glycogen is a large, branched polymer of glucose residues and serves as the body's storage form of this sugar. Alpha-1,4-glycosidic bonds link most of the glucose residues in glycogen. Branches are created by alpha-1,6-glycosidic bonds, of which there is one in about ten residues. The glycogen macromolecule also contains the enzymes that catalyze the synthesis and degradation of glycogen and some of the enzymes regulating these processes. The presence of glycogen greatly increases the amount of carbohydrate that is immediately available between meals and during muscular contraction. The two major sites of glycogen storage are liver and skeletal muscles. The concentration of glycogen is higher in the liver but, due to its much greater mass, skeletal muscle stores more total glycogen. Glycogen metabolism in skeletal muscle plays a major role in the control of blood glucose homeostasis by the pancreatic hormone insulin, the most important regulator of blood glucose levels. Understanding the process by which insulin regulates glycogen accumulation in muscle is relevant to both insulin resistance and restoration of carbohydrate stores following exercise.

Glucose Disposal

Glucose is a relatively polar molecule requiring a transporter for facilitated diffusion across the plasma membrane. Two isoforms of the facilitative glucose

transporter family, GLUT1 and GLUT4, are expressed in skeletal muscle (Douen et al., 1990). GLUT1 resides primarily in the plasma membrane where it likely plays a role in basal, but not insulin-stimulated glucose transport (Douen et al., 1990). Conversely, GLUT4 is predominantly located intracellularly, but is translocated to the plasma membrane by the action of insulin (Douen et al., 1990) and contractile activity (Douen et al., 1990; Hirshman et al., 1990). The greater number of GLUT4 transporters incorporated into the plasma membrane during translocation, the greater the rate of glucose transport (Etgen et al., 1996; Lund et al., 1993). The hormone insulin is released from pancreatic beta cells in response to rising blood glucose resulting in an indirect coupling of glucose appearance and disappearance rates in the circulation. A stable blood glucose concentration depends on this balance.

Glucose transport is the penetration of glucose through the plasma membrane while glucose uptake is the clearance of glucose from the extracellular space and involves the processes of glucose transport and intracellular glucose disposal. Once a glucose molecule is transported into a cell, it is either phosphorylated by hexokinase to G-6-P or counter-transported back to the extracellular compartment. Muscle tissue cannot dephosphorylate glucose because it lacks glucose-6-phosphatase. Therefore, phosphorylation effectively commits the G-6-P to metabolism within the cell, as the negatively charged phosphate does not permit the G-6-P to cross the plasma membrane. Insulin binding to its receptor results in translocation of GLUT4 to, as well as its insertion into, the plasma membrane. This process has not been fully elucidated. However, it is known that

the greater number of GLUT4 transporters that are incorporated into the plasma membrane in response to insulin, the greater the rate of glucose transport (Etgen et al., 1996; Lund et al., 1993; Wilson and Cushman, 1994). Additionally, an increase in GLUT4 protein content is generally associated with increased insulin responsiveness (Henriksen et al., 1990; Kern et al., 1990). In skeletal muscle, the majority of G-6-P formed during insulin-stimulated glucose uptake is rapidly converted to glycogen or oxidized (Ivy and Holloszy, 1981; Lillioja et al., 1986). Typically, the rate of phosphorylation will exceed the rate of transport, maintaining a low intracellular free glucose concentration and providing a concentration gradient essential for net inward glucose transport. However, hexokinase activity is allosterically inhibited by its product, G-6-P. Therefore, if G-6-P is not rapidly removed, glucose disposal can become rate limiting and result in a reduction in the rate of glucose uptake and clearance of glucose from the circulation.

Glycogenolysis

Glycogen breakdown occurs by orthophosphate cleavage to a phosphorylated sugar, glucose-1-phosphate (G-1-P) (Cori and Cori, 1947). This reaction is catalyzed by the enzyme glycogen phosphorylase. Phosphorylase catalyzes the sequential removal of glycosyl residues from the non-reducing end of the glycogen molecule. The glycosidic linkage between C-1 of the terminal residue and C-4 of the adjacent residue is split by orthophosphate. Specifically,

the bond between the C-1 carbon atom and the glycosidic oxygen atom is cleaved by orthophosphate, and the alpha configuration at C-1 is retained.

Glycogen is not degraded by phosphorylase alone as the alpha-1,6-glycosidic bonds at the branch points are not susceptible to cleavage by phosphorylase. Phosphorylase stops cleaving alpha-1,4-linkages when it reaches a terminal residue four residues away from an existing branch. A transferase exposes the alpha-1,6 branch residue to the action of a third degradative enzyme, an alpha-1,6-glucosidase (debranching enzyme), that hydrolyzes the alpha-1,6-glycosidic bond liberating a free glucose molecule. Thus, the transferase and the debranching enzyme convert the branched structure into a linear one, allowing for further cleavage by phosphorylase. The G-1-P formed in the phosphorolytic cleavage of glycogen is converted into G-6-P by phoshphoglucomutase.

Glycogen Synthesis

In the synthesis of glycogen, intracellular glucose undergoes several modifications to generate uridine diphosphate (UDP)-glucose (Leloir et al., 1971). This reaction takes place in three sequential steps. First, glucose is phosphorylated by hexokinase to generate G-6-P, which is then converted to G-1-P by phosphoglucomutase. Finally, UDP-glucose is synthesized from G-1-P and uridine triphosphate in a reaction catalyzed by UDP-glucose pyrophosphorylase. The C-1 carbon of the glycosyl unit of UDP-glucose is considered “activated” because its hydroxyl group is esterified to the diphosphate moeity of UDP.

New glucose units are added to the non-reducing terminal residues of glycogen. The UDP-glucose is transferred to the hydroxyl group at a C-4 terminus of glycogen forming an alpha-1,4-glycosidic linkage. In this elongation reaction, the growing glycogen macromolecule's terminal hydroxyl group displaces UDP. This reaction is catalyzed by glycogen synthase, which can add glucose residues only if the polysaccharide chain already contains more than four residues. Thus, glycogen synthesis requires a primer, which is formed by a different synthase.

Glycogen synthase catalyzes only the synthesis of alpha-1,4-linkages. A branching enzyme is needed to form the alpha-1,6-linkages that make glycogen a branched polymer. Branching is important because it increases the solubility of glycogen. Furthermore, branching creates a large number of non-reducing terminal residues, which are the sites of action of glycogen phosphorylase and synthase. Thus, branching increases the rate of glycogen synthesis and degradation.

Branching occurs after a number of glucose residues are joined in alpha-1,4-linkage by glycogen synthase. The breakage of an alpha-1,4-link and the formation of an alpha-1,6-link creates branching. A block of residues, typically seven in number, is transferred to a more interior site of the macromolecule. The branching enzyme that catalyzes this reaction is quite precise. The block of seven residues must include the non-reducing terminus and come from a chain of at least eleven residues long. In addition, new branch points are at least four residues away from a pre-existing branch.

The energy yield from the breakdown of glycogen is highly efficient. About 90% of the residues are phosphorylitically cleaved to G-1-P, which is converted at no metabolic cost into G-6-P. The other 10% are branch residues, which are hydrolytically cleaved. One ATP is then used to phosphorylate each of these glucose molecules to G-6-P. The complete oxidation of G-6-P yields 37 molecules of ATP and storage consumes one ATP per G-6-P.

Glycogenin and Pro- and Macroglycogen

The primer for glycogen synthesis is an autoglucosylating protein called glycogenin. This protein is capable of catalyzing the addition of glucosyl units to its Try-194 binding site. In skeletal muscle glycogenin and glycogen synthase are complexed with the glycogen macromolecule under resting conditions with no detectable free deglycosylated glycogenin. In rat skeletal muscle, glycogenin varies with fiber type with type I fibers having the greatest content and activity (Hansen, 1999). Interestingly, muscle glycogenin content and activity are not affected by endurance exercise training (Hansen, 1999). Glycogenin may play an important role in glycogen metabolism. However, the mechanisms remain unclear. Hansen (1999) has evaluated three possible sites of regulation on the protein itself including interaction with glycogen synthase, a phosphorylation site, and regulation by glucose metabolites. Glycogenin and glycogen synthase disassociate when skeletal muscle is exposed to prolonged electrical stimulation or epinephrine with reassociation of the glycogen-free glycogenin and glycogen synthase being time dependent (Smythe et al., 1990). It has been suggested that

this reassociation may be influenced by neural and hormonal factors, which are known to regulate glycogen synthesis (Shearer et al., 1999). Regulation of glycogenin by glucose metabolites or its putative interaction with GLUT4 have been suggested but not investigated. However, phosphorylation of the glycogenin protein has been demonstrated in skeletal muscle (Lomako and Whelan, 1988) but the possible regulatory role is unknown. Transcriptional or translational regulation of glycogenin may also exist.

There are two forms of glycogen formed from a single glycogenin primer, proglycogen and macroglycogen (Alonso et al., 1995; Lomako et al., 1991; Lomako et al., 1993). As more glucosyl units are added to a proglycogen molecule it expands to a macroglycogen molecule (Alonso et al., 1995). These two types of glycogen differ in size, carbohydrate and solubility. Macroglycogen contains more carbohydrate and is larger than proglycogen. Macroglycogen is soluble in perchloric acid while proglycogen precipitates. In human skeletal muscle with normal glycogen levels, macroglycogen accounts for ~25% of the total glycogen concentration (Adamo et al., 1998; Jansson et al., 1981). The proportion of pro- and macroglycogen varies widely in different muscle fiber types (Alonso et al., 1995). Because there are no free glycogenin molecules in skeletal muscle at rest, accumulation of proglycogen would require synthesis of more glycogenin protein primers while macroglycogen could accumulate simply by the addition of glycosyl units. Regulation of synthesis and degradation of the glycogen fractions is not clearly understood. Furthermore, the metabolic

significance of glycogenin as well as the distinct glycogen pools requires more research.

Glycogen Synthase

Glycogen synthase is controlled by multisite phosphorylation and by several allosteric effectors, of which the activator G-6-P is most important. Of the 7 carboxy-terminal phosphorylation sites, Ser^{640} (site 3a) and Ser^{644} (site 3b) and NH_2-terminal Ser^{7} (site 2) and Ser^{10} (site 2a) are important regulation locales (Skurat et al., 2000). In general, phosphorylation decreases glycogen synthase activity, causing an increase in the apparent K_m for the substrate UDP-glucose and an increase in the K_a for G-6-P. The extent of inactivation depends on the sites phosphorylated and metabolic effector concentrations. G-6-P, for example, is able to fully activate even highly phosphorylated forms of the enzyme (Villar-Palasi and Guinovart, 1997). The hormone insulin, for example, activates glycogen synthase by promoting dephosphorylation of the enzyme, resulting in an increase in G-6-P sensitivity of glycogen synthase, i.e., a decrease in the concentration of G-6-P required to activate the enzyme (Villar-Palasi, 1991). The enzymes of glycogen metabolism are dephosphorylated by glycogen-bound protein phosphatase-1 (PP-1). In addition, PP-1 is capable of dephosphorylating all of the sites on glycogen synthase. The existence of differential regulation or two isoforms of glycogen synthase for pro- and macroglycogen has been speculated but not demonstrated.

Regulation of Glycogen Metabolism by Insulin

Insulin regulates the synthesis of glycogen at two steps, first by controlling the transport and uptake of glucose into the muscle cell (Narahara et al., 1960), and second, by regulating the phosphorylation and activating states of enzymes involved in the synthesis and degradation (Villar-Palasi and Larner, 1960). In recent years, significant advances have been made to understand the mechanisms of insulin-induced activation of glycogen synthesis. However, the precise steps involved in this process remain uncertain.

Insulin causes a decrease in the serine phosphorylation of glycogen synthase leading to its activation (Parker et al., 1983; Roach, 1991; Lawrence, 1994). This decrease could be accomplished either by activating PP-1 which dephosphoylates glycogen synthase and/or by inhibiting the protein kinases, which catalyze the phosphorylation of glycogen synthase. For example, insulin activates PP-1 activity in skeletal muscle (Begum, 1995a; Newgard et al., 2000; Oliver et al., 1988; Oliver et al., 1990; Srinivasan et al., 1994; Toth et al., 1991). Since PP-1 can dephosphorylate all the sites of glycogen synthase, its activation by insulin has been attributed to a decrease in the phosphorylation of glycogen synthase (Begum, 1995b; Hubbard and Cohen, 1993). However, which form of PP-1 is involved and the mechanism of activation is unknown (Walker et al., 2000).

Glycogen Synthase Kinase-3

Recent research suggests that insulin activates muscle glycogen synthase in part by decreasing the activity of glycogen synthase kinase-3 (GSK-3) (Cross et al., 1997; Ueki et al., 1998). This enzyme is constitutively active in the basal state and is inhibited by Ser^{21} and Ser^{9} phosphorylation in response to insulin and other growth factors in various cell types (Hughes et al., 1992; Welsh and Proud, 1993; Cross et al., 1994). Following site 5 phosphorylation of glycogen synthase by casein kinase II, GSK-3 phosphorylates a series of serine residues (sites 4, 3c, 3b, and 3a) at the carboxy terminus of glycogen synthase and inactivates it (Lawrence and Roach, 1997; Parker et al., 1983). *In vitro*, GSK-3 phosphorylates two (3b and 3c) of the four sites (3b, 3c, 2, and 2a) on glycogen synthase, which also become dephosphorylated in response to insulin (Moxham et al., 1996). Recently, PKB/Akt has emerged as the most potential protein kinase catalyzing the phosphorylation and inactivation of GSK-3 in skeletal muscle (Cross et al., 1995). The sites of PKB/Akt phosphorylation of GSK-3 are reported as the same sites phosphorylated in response to insulin *in vivo* (Cross et al., 1995). GSK-3, in turn, has been implicated in feedback inhibition of IRS-1-facilitated PI3-kinase activity (Eldar-Finkelman and Krebs, 1997; Summers et al., 1999). Thus, PKB/Akt may play a pivotal role in both insulin regulation of glucose transport and glycogen synthase activity. However, several other insulin-stimulated protein kinases including epidermal growth factor, c-Jun NH_2-terminal kinase, mitogen-activated/90 kDa ribosomal kinase (p^{90rsk}) and 70 kDa ribosomal S6 kinase (p^{70rsk}) have been shown to catalyze the phosphorylation and inactivation of GSK-3 *in*

vitro (Moxham et al., 1996; Sutherland et al., 1993; Sutherland and Cohen, 1994). Therefore, the existence of redundant GSK-3 inactivation mechanisms may facilitate enhanced activity of glycogen synthase. However, a study using specific inhibitors of p^{70rsk} and p^{90rsk} demonstrated that these kinases might not catalyze the *in vivo* phosphorylation and inhibition of GSK-3 activity (Cross et al., 1994). Skurat et al. (2000) recently demonstrated that GSK-3 inactivation is not essential for insulin-stimulated glycogen synthase activation in rat fibroblasts. In addition to indirectly inactivating GSK-3, insulin also increases the dephosphorylation of glycogen synthase by activating a glycogen-associated form of PP-1 (Newgard et al., 2000; Walker et al., 2000).

Upon cessation of exercise, a PKB/Akt independent inhibition of GSK-3 has been observed in skeletal muscle *in vivo* (Markuns et al., 1999). However, this deactivation does not appear to be responsible for the prolonged activation (i.e., 4 h post-exercise) of glycogen synthase during the insulin-dependent phase of post-exercise glycogen resynthesis as GSK-3 activity is similar in rested and exercised muscle (Wojtaszewski et al., 2000). How GSK-3 is inhibited or possibly PP-1 activated, for that matter, by previous exercise is currently unknown but may be related to increased c-Jun NH_2-terminal kinase activity (Aronson et al., 1997; Moxham et al., 1998; Sherwood et al., 1999).

PI3-Kinase and PKB/Akt

Protein kinase B is the cellular homolog of the retroviral Akt (Bellacosa et al., 1991; Coffer and Woodgett, 1991; Jones et al., 1991). PKB/Akt is rapidly

activated through insulin-mediated phosphorylation (Alessi et al., 1996; Cross et al., 1995). PI3-kinase has been implicated in the activation of PKB/Akt as the phosphorylation of PKB/Akt is blocked by wortmannin, an inhibitor of PI3-kinase (Alessi et al., 1996). PI3-kinase activity is stimulated by insulin (Ruderman et al., 1995) as well as many other stimuli (Toker and Cantley, 1997; Vanhaesebroek et al., 1997). The SH2 domain of the PI3-kinase p^{85} subunit interacts with tyrosine phosphorylated insulin receptor substrates 1 and 2 (IRS-1, IRS-2). This interaction results in the stimulation of the catalytic activity of the p^{110} subunit of PI3-kinase (Cheatham and Kahn, 1995). The PtdIns 3,4-biphosphate and PtdIns 3,4,5-triphosphate lipid products of the PI3-kinase reaction have been shown to promote association of 3-phosphoinositide-dependent kinase-1 (PDK-1) and PKB/Akt whereby PDK-1 then activates PKB/Akt via Ser^{473} and Thr^{308} phosphorylation (Alessi and Cohen, 1998). Activation of PKB/Akt has been implicated in insulin-stimulated GLUT4 translocation and glycogen synthase activation (Cheatham and Kahn, 1995). Turinsky and Damrau-Abney (1999) recently demonstrated that the increase in insulin-stimulated rat muscle glucose uptake is associated with an increased activity of PKB/Akt-1 as opposed to PKB/Akt-2 or PKB/Akt-3. This association was most pronounced in the primarily slow twitch soleus but was apparent in the plantaris and gastrocnemius muscles as well.

A few studies have demonstrated that insulin-stimulated PI3-kinase activity is not increased immediately post-exercise (Goodyear et al., 1995; Whitehead et al., 2000; Wojtaszewski et al., 1997). However, recent evidence for

increased insulin-stimulated PI3-kinase activity following exercise exists (Chibalin et al., 2000; Derave et al., 2000; Howlett et al., 2002; Kawanaka et al., 2000; Wadley et al., 2001). Chibalin et al. (2000) observed an increase in rat muscle insulin-stimulated IRS-1- and IRS-2-associated PI3-kinase activities 16 h following an acute exercise bout. The increase in insulin-stimulated IRS-2-associated PI3-kinase activity following exercise has recently been confirmed by Howlett et al. (2002). Wadley et al. (2001) demonstrated an increased expression of PI3-kinase mRNA 3 h following a single bout of exercise in humans. Expression of PI3-kinase mRNA was not elevated immediately after exercise or following endurance exercise training in this study. PI3-kinase activity was not presented in this study. Studies by Derave et al. (2000) and Kawanaka et al. (2000) further demonstrated that the increased insulin-stimulated PI3-kinase activity following exercise is not affected by muscle glycogen content.

During exercise, PKB/Akt is not essential for the regulation of muscle GLUT4 translocation, glucose transport (Lund et al., 1998) or glycogen synthase activity (Sakamoto et al., 2002). Recent studies have demonstrated that muscle PKB/Akt activity is not increased following treadmill running (Nader and Esser, 2001; Markuns et al., 1999) or swimming (Derave et al., 2000) in rats. These findings are supported by studies which involved electrically induced contractions *in vitro* (Brozinick and Birnbaum, 1998; Lund et al., 1998; Whitehead et al., 2000) and *in situ* (Sherwood et al., 1999). Using the *in vitro* isolated rat muscle preparation, Lund et al. (1998) observed that insulin but not contraction resulted in an increase in PKB/Akt activity. When insulin and contraction were combined,

the resultant PKB/Akt activity was not different than following insulin alone. Furthermore, insulin-stimulated PKB/Akt activity is not significantly higher 16 h following acute exercise in rats (Chibalin et al., 2000), while in human muscle, insulin-stimulated PKB/Akt activity was not increased 4 h following an acute bout of one-legged cycling (Wojtaszewski et al., 2000).

Despite the findings from the above studies, sufficient evidence for a PKB/Akt increase in response to contractile activity exists (Derave et al., 2000; Kawanaka et al., 2000; Nader and Esser, 2001; Sakamoto et al., 2002, Thorell et al., 1999; Turinsky and Damrau-Abney, 1999). Thorell et al. (1999) assessed PKB/Akt activity in human muscle biopsies following 60 min of moderate-intensity cycle exercise. PKB/Akt Ser^{473} phosphorylation was increased by 280% above resting values immediately upon exercise completion. In addition, when a 2 h euglycemic-hyperinsulinemic clamp was performed following exercise, PKB/Akt Ser^{473} phosphorylation was increased 1,000% above resting values. Nader and Esser (2001) observed an increase in rat tibialis anterior PKB/Akt phosphorylation immediately after 20 min of *in situ* electrical stimulation via the sciatic nerve. This increased activity was evident following high frequency as well as low frequency stimulation. Interestingly, treadmill running exercise failed to elicit an increase in PKB/Akt immediately following exercise in this study (Nader and Esser, 2001). Sakamoto et al. (2002) demonstrated an *in situ* contraction-mediated increase in the activities of all three PKB/Akt isoforms. Maximal activation of the kinase isoforms occurred after 2.5 min of contractions and had returned to baseline levels by 15 min. Three isoforms of PKB/Akt have

been identified. The primary insulin-stimulated isoform in rodent muscle is PKB/Akt-1 (Walker et al., 1998). Turinsky and Damrau-Abney (1999) recently observed an increased in PKB/Akt-1 activity throughout a 25 min *in situ* high frequency electrical stimulation protocol. This group also observed an insulin-mediated increase in the activity of this isoform. The activities of PKB/Akt-2 and PKB/Akt-3 were not altered by the stimulation protocol. Two recent studies using rats have demonstrated that PKB/Akt activity increased following swimming exercise (Derave et al., 2000; Kawanaka et al., 2000). Derave et al. (2000) demonstrated that PKB/Akt activity was increased when previously exercised muscle was exposed to supraphysiologic insulin concentrations *in situ*. Using an isolated *in vitro* muscle preparation, Kawanaka et al. (2000) also observed an increase in insulin-stimulated PKB/Akt in previously exercised muscle. Both of these studies, however, demonstrated that elevated muscle glycogen content was associated with an attenuation of insulin-stimulated increases in PKB/Akt activity.

The ability of exercise to activate skeletal muscle PKB/Akt may by mode- and isoform-specific. In addition, whether or not exercise enhances insulin-stimulated PKB/Akt activity may depend on the insulin concentration as well as muscle glycogen content. Regardless, further research in this area is certainly warranted.

Exercise and Glycogen Metabolism

During prolonged strenuous exercise, carbohydrate is a required fuel for sustained muscle contraction. Immediate sources of carbohydrate are muscle

glycogen and blood glucose. Early in exercise, muscle glycogen is the preferred source of carbohydrate. However, as exercise continues and the muscle glycogen levels decline, the rate of glycogenolysis also declines and there is an increased reliance on blood glucose to support the working muscle's carbohydrate requirements (Ahlborg et al., 1967; Bergström et al., 1967; Hermansen et al., 1967). To accommodate this increased reliance on blood glucose, GLUT4-containing vesicles are translocated to the plasma membrane (Kristiansen et al., 1997). Thus, glycolysis and glucose oxidation may remain relatively constant as long as there is no major decrease in the blood glucose concentration. However, the decline in muscle glycogen results in perception of fatigue and once depleted, necessitates termination of exercise or a significant reduction in exercise intensity (Ahlborg et al., 1967; Bergström et al., 1967; Hermansen et al., 1967). In addition, it has been found that aerobic endurance performance is directly related to the initial muscle glycogen stores (Ahlborg et al., 1967; Hermansen et al., 1967; Hultman, 1967). Thus, following prolonged exercise, replenishment of muscle glycogen to within a normal range is an essential component of the recovery process.

During exercise the interaction of blood glucose and muscle glycogen utilization is complicated by fat metabolism. Alterations in plasma free fatty acid (FFA) levels influence muscle glycogenolysis during exercise. For example, inhibition of FFA mobilization by nicotinic acid lowers plasma FFA and increases muscle glycogen use during exercise (Bergström et al., 1969). In contrast, increased plasma FFA availability has been shown to reduce the rate of muscle

glycogen utilization during exercise (Costill et al., 1977; Dyck et al., 1996; Hickson et al., 1977; Rennie et al., 1976; Vulkovich et al., 1993) via post-transformational regulation of glycogen phosphorylase activity (Dyck et al., 1996). Following exercise, elevated free fatty acid concentrations are associated with impaired muscle insulin action (Schulch and Kipnis, 1965).

Post-Exercise Insulin Action

Following exercise, the synthesis of muscle glycogen occurs as a bi-phasic response with the first rapid phase being insulin-independent and the second slower phase being insulin-dependent (Ivy, 1977; Mæhlum et al., 1977; Price et al., 1994). During the insulin-independent phase the rate of glycogen synthesis is very rapid, but lasts only 30 to 45 minutes (Price et al., 1994). During the insulin-dependent phase, synthesis remains negligible unless there is an increase in glucose availability and plasma insulin concentration (Ivy, 1977; Price et al., 1994).

Previous muscle contraction results in an increase in insulin stimulation of glucose transport (Cartee et al., 1989; Garetto et al., 1984; Holloszy and Narahara, 1965; Ren et al., 1994; Richter et al., 1984). This is of benefit as energy expenditure is coupled to recovery by partitioning substrate toward previously active muscle. When muscle contraction ceases, the energy requirement of the muscle declines rapidly and glycolysis is substantially reduced. Glucose transport, however, remains elevated due to an increased number of glucose transporters on the plasma membrane (Goodyear et al., 1990). The combination of enhanced

glucose transport, but low glycolytic flux results in an elevation of G-6-P (Bloch et al., 1994). The increase in G-6-P stimulates glycogen synthesis by activating glycogen synthase allosterically, increasing susceptibility to attack by PP-1 following exercise (Villar-Palasi and Guinovart, 1997). Availability of PP-1 is increased due to its simultaneous release with glycogen synthase from the glycogen macromolecule during glycogenolysis (Hubbard and Cohen, 1993). Results from a recent study by Aschenbach et al. (2001) indicate that PP-1 activation is essential for glycogen synthase regulation during exercise. In addition, contractile activity increases the mitogen-activated kinase pathway (Hayashi et al., 1999). As mentioned in the previous section, certain components of this pathway may inactivate GSK-3. As the residual effects of muscle contraction subside, the membrane bound GLUT4 returns to an intracellular site (Derave et al., 1999; Etgen et al., 1996) and glucose transport (Hespel and Richter, 1990) and intracellular G-6-P levels decline (Bloch et al., 1994). However, glycogen synthesis continues, albeit at a considerably slower rate, due to increased insulin action in muscle.

When carbohydrate is provided during the recovery process, the rate of glycogen synthesis is increased further and glycogen supercompensation occurs (Bergström et al., 1967; Ivy, 1991). Carbohydrate feeding most likely stimulates glycogen supercompensation by increasing plasma glucose and insulin concentrations, which in turn enhance the effects of post-exercise insulin action (e.g., substrate availability, G-6-P and insulin-mediated activation of glycogen synthase).

The regulation of the slower, insulin-dependent phase of post-exercise glycogen recovery is not well understood. However, the activity of glycogen synthase is increased following exercise due to high levels of G-6-P and low glycogen concentration, both being potent activators of the enzyme (Bloch et al., 1994). This enhanced insulin action persists until muscle glycogen levels are replenished (Price et al., 1994). The mechanisms responsible for the reversal of exercise-induced, insulin-dependent phase of glycogen synthesis are not clear. As muscle glycogen stores are restored, this causes a reduction in insulin-stimulated muscle glucose transport (Cartee and Holloszy, 1990). Increased muscle glycogen content also lowers the activity of glycogen synthase (Danforth, 1965; Laurent et al., 2000). Thus, a major pathway of blood glucose disposal is down regulated.

Pro- and Macroglycogen Accumulation Following Exercise

It is not known whether pro- and macroglycogen are catabolized at different rates during exercise; however, it is known that both are utilized (Asp et al., 1999). Following exercise, synthesis of both pro- and macroglycogen is sensitive to carbohydrate availability. Adamo et al. (1998) demonstrated that during the 48 h following exhaustive exercise net synthesis rates for pro- and macroglycogen were higher when high carbohydrate was provided compared to a low carbohydrate trial. The net synthesis rate of proglycogen decreased in the high carbohydrate group throughout the recovery period and was not different than low carbohydrate group from 24 to 48 h. Conversely, the net synthesis rate of macroglycogen remained higher in the high carbohydrate group compared to

the low carbohydrate group throughout the 48 h recovery period. These results suggest that proglycogen is predominantly synthesized during the early phase of glycogen recovery when blood glucose and insulin concentrations are elevated. This accumulation would require more glycogenin protein suggesting additional regulation by insulin, i.e., increased protein synthesis or decreased protein degradation. However, the slower but consistently elevated macroglycogen synthesis rate accounts for the majority of the glycogen formed as total glycogen is elevated to above normal levels. This finding is in agreement with other studies of macroglycogen accumulation in human (Adamo and Graham, 1998; Asp et al., 1999; Jansson et al., 1981) and rat muscle (Huang et al., 1997).

In the study by Adamo et al. (1998) the net glycogen synthesis rate during the low carbohydrate trial was approximately 6 mmol • kg dw^{-1} • h^{-1}, which is similar to previously reported results (Ivy et al., 1988). Furthermore, in the high carbohydrate group, the sum of the pro- and macroglycogen synthesis rates during the first 4 h after exercise (19 mmol • kg dw^{-1} • h^{-1}) was similar to the total glycogen synthesis rate observed by Ivy et al. (1988).

The different time courses of pro- and macroglycogen storage suggest different regulation of the glycogen fractions. Although a putative differential glycogen synthase regulation and/or isoform for pro- and macroglycogen formation has not been demonstrated, the activation state of glycogen synthase is very different during the two phases of post-exercise glycogen restoration. For example, during the initial, rapid, insulin-independent phase the elevated G-6-P concentration allosterically activates glycogen synthase (Bloch et al., 1994). This

phase is associated primarily with proglycogen accumulation. Following glycogen restoration to normal levels, i.e., during glycogen supercompensation, when macroglycogen formation is predominant, glycogen synthase activity is attenuated (Laurent et al., 2000).

Asp et al. (1999) has recently reported that a greater fraction of muscle macroglycogen than proglycogen is used during prolonged, muscle damaging exercise in humans. Following this type of exercise, pro- and macroglycogen restoration were delayed despite provision of a high carbohydrate diet. This delay was particularly apparent in type I and type IIA fibers compared to type IIB fibers. This fiber type difference suggests that the fibers used most during the marathon accumulate post-exercise glycogen differently than non-recruited fibers (Sherman et al., 1983). The accumulation of macroglycogen was impaired despite both a high carbohydrate diet and normal concentrations of proglycogen precursor. The post-exercise glycogen accumulation findings of Asp et al. (1999) differ from the high carbohydrate trial of Adamo et al. (1998) suggesting that the pro- and macroglycogen pools are regulated differently following exercise that results in muscle damage such as the marathon.

Skeletal Muscle Glycogen Levels and Glucose Uptake

It has been demonstrated that muscle glycogen concentration affects both membrane glucose transport and intracellular glucose metabolism. Fell et al. (1982) showed that at the same submaximal insulin concentration the rate of post-exercise glucose uptake was greater in muscles in which glycogen content was

lowered by carbohydrate restriction compared to muscles in which glycogen content was raised with carbohydrate feeding. In a recent *in vitro* study by Jensen et al. (1997), epitrochlearis muscle basal and insulin-stimulated glucose uptakes were inversely related to initial glycogen content. A decline in plasma membrane permeability during post-exercise recovery appears to be inversely related to the muscle glycogen content (Cartee et al., 1989). This raises the possibility that glycogen may have some control over the number of transporters that can be actively associated with the plasma membrane. Evidence for this possibility is provided by the study of Etgen et al. (1996). This study compared the effect of muscle contractions on glucose transport, cell-surface GLUT4 labeling, and glycogen concentration in soleus and epitrochlearis muscle preparations. Using the same stimulation protocol for both muscles, it was found that the contraction-induced increase in glucose transport was significantly higher and muscle glycogen content significantly lower in the epitrochlearis when compared to the soleus. Furthermore, the increase in transport in both muscles was highly associated with the cell-surface GLUT4 concentration (Etgen et al., 1996). Using the perfused rat hindlimb procedure, Derave et al. (1999) recently observed that the contraction-induced increase in muscle glucose transport and cell-surface GLUT4 content were negatively correlated with the initial glycogen concentration. These results are supported by other perfused rat hindlimb studies, which suggest an inverse relationship between muscle glycogen levels and insulin-stimulated glucose uptake (Hespel and Richter, 1990). Alterations in

muscle G-6-P levels, hexokinase activity and intracellular free glucose may also influence muscle glucose uptake during glycogen accumulation.

Evidence for Glucose Transport as Rate Limiting for Glycogen Synthesis

The relative importance of glucose transport and glycogen synthase in controlling the rate of glycogen synthesis remains unclear. Under basal conditions, however, most evidence suggests that glucose transport is the principle rate-determining step for glucose metabolism, as there is little free glucose in resting skeletal muscle fibers (Ziel et al., 1988). Recently, the importance of glucose transporters in determining the rate of glycogen synthesis has been demonstrated leading to widely held view that most of the control is at the level of the transporter. Utilizing metabolic control analysis, Shulman et al. (1995) demonstrated that the rate of glucose entry into the muscle cell plays a dominant role in glycogen accumulation. In addition, studies with transgenic mice overexpressing the skeletal muscle glucose transporters, GLUT1 and GLUT4, have provided evidence that increasing glucose transport is sufficient to increase glycogen synthesis (Hansen et al., 1995; Ren et al., 1993; Tsao et al., 1996). Basal muscle glycogen content of the transgenic animals was markedly increased, although the activation-state of glycogen synthase in the transgenic animals was not different from control muscles (Ren et al., 1993). In addition, transgenic mice overexpressing GLUT1 exhibit increased glycogen synthesis after exercise (Ren et al., 2000). Furthermore, mice heterozygous for a disrupted GLUT4 allele, expressing 50% of the wildtype level of GLUT4, exhibited impaired glycogen

synthesis during euglycemic-hyperinsulinemic clamps (Rosetti et al., 1997). Take together, these results suggest that glucose transport is rate limiting for glycogen synthesis. However, results from animals with genetically modified expression of glucose transporters do not exclude the possibility that activation of glycogen synthase by insulin has an important role in the stimulation of glycogen synthesis. In addition, glucose transport control of glycogen formation may not be valid under all conditions *in vivo* and evidence for shared control, i.e., transport and glycogen synthase, has been provided (Bogardus et al., 1984; Jucker et al., 1999; Kashiwaya et al., 1994; Kubo and Foley, 1986; Schulz, 1998; Shulman et al., 1995; Smith and Lawrence; 1984).

Evidence for Glycogen Synthase as Rate Limiting for Glycogen Synthesis

In skeletal muscle evidence for glycogen synthase control of the rate of glycogen synthesis has been provided by a number of investigators. This may be true under certain physiologic conditions. For example, Smith and Lawrence (1984) demonstrated that following denervation of rat hemidiaphragms, a decrease in the ability of insulin to activate glycogen synthase preceded the inhibition of the hormone to stimulate glucose transport. Recent work using metabolic control analysis has illustrated that the control of glucose uptake is distributed among glucose transport and phosphorylation, glycogen synthesis and glycolysis (Jucker et al., 1999; Kashiwaya et al., 1994; Shulman et al., 1995). Through metabolic control analysis of glycogen synthesis, Schulz (1998) concluded that the control of glycogen synthesis is distributed among different

metabolic steps, which exert varying influences depending on the physiologic conditions, i.e. hormone and substrate concentrations. Jucker et al. (1999) also assessed metabolic control of *in vivo* glucose disposal by glucose transport, glycogen synthase and glycolysis, demonstrating that all three steps affect glucose disposal. In addition to varied hormonal and substrate levels, fiber type differences in insulin sensitivity (James et al., 1985) may account for differences in the distribution of control. For example, it has been shown that the regulation of glycogen synthase is different in fast versus slow twitch muscle after a glucose load (Sugden et al., 1997).

Recent Studies Using Transgenic Mice Overexpressing Glycogen Synthase

Results from metabolic control analysis are further supported by the findings of Manchester et al. (1996) and Azpiazu et al. (2000). These investigators have recently utilized a transgenic mouse overexpressing glycogen synthase to assess control of insulin-stimulated glycogen accumulation from a novel perspective. Glycogen synthase activity was increased 10-fold in transgenic muscles. Associated with the elevated levels of synthase were glycogen concentrations as much as 5-fold higher than found in wildtype muscles (Manchester et al., 1996). Several lines of evidence from the study of Manchester et al. (1996) suggest that the increase in glycogen was due to the increase in glycogen synthase, and not due to increased glucose transport. For example, UDP-glucose concentrations were lower in transgenic muscle *in vivo*, which may be indicative of glycogen synthase control of glycogen intermediate

concentrations. In addition, levels of GLUT4 were the same or slightly lower in transgenic compared to wildtype muscle. Azpiazu et al. (2000) extended these findings by measuring the insulin-stimulated rates of 2-deoxyglucose uptake in diaphragm, extensor digitorum longus and soleus muscles. In muscles incubated *in vitro*, basal and insulin-stimulated rates of glucose uptake in the transgenic and wildtype muscles were not different. In response to insulin, the increases in glucose uptake from basal were 8-fold in diaphragm, 2-fold in extensor digitorum longus and soleus. In muscles incubated *in vitro* with a maximally effective concentration of insulin (20 mU • ml^{-1}) or without insulin, the rate of ^{14}C-glucose incorporation into glycogen was 2- to 2.5-fold higher in transgenic muscle compared to wildtype muscle (Azpiazu et al., 2000). For example, a 24–fold insulin-stimulated increase (above basal) in glucose incorporation into glycogen was observed in the transgenic diaphragm. The increased incorporation in this muscle could not be accounted for by either the insulin-stimulated increase in glucose uptake (8-fold) or increase in glycogen synthase activity ratio (4-fold) alone. This suggests insulin-stimulated increases in both processes may account for the enhanced glycogen incorporation. With no difference in insulin-stimulated glucose uptake between the wildtype and transgenic muscle, the enhanced incorporation into glycogen in transgenic muscle is likely due to a greater percentage of intracellular G-6-P going to glycogen synthesis as opposed to glycolysis, the hexoasamine pathway, or oxidation. Thus, overexpression of glycogen synthase could potentially facilitate a repartitioning of intracellular glucose intermediates. Azpiazu et al. (2000) concluded that their results were

evidence for a shared control of insulin-stimulated glycogen synthesis involving glycogen synthase as well as glucose transport. These results concur with the recent findings of Jucker et al. (1999). Using metabolic control analysis, this group determined that during insulin stimulation, the majority of control of glucose uptake is by glucose transport and glucose phosphorylation. However, glycogen synthesis and glycolysis were also determined to control glucose uptake, albeit to a lesser extent.

Insulin-stimulated glucose uptake was not different in wildtype and transgenic muscle in the study by Azpiasu et al. (2000). In addition, Manchester et al. (1996) demonstrated that the glycogen content of the transgenic muscles is several-fold higher than wildtype. Thus, the lack of difference in insulin-stimulated glucose uptake between the transgenic and wildtype muscle may have been affected by the higher glycogen content of the transgenic animals. In this regard, it has been demonstrated that the rate of insulin-stimulated glucose uptake in muscle is inversely related to glycogen concentration (Derave et al., 2000; Fell et al., 1982; Jensen et al., 1997; Kawanaka et al., 2000). Recently, Derave et al. (2000) demonstrated that elevated muscle glycogen levels were associated with impaired insulin signaling (PKB/Akt activity) and translocation of GLUT4 transporters. Kawanaka et al. (2000) also observed that glycogen-supercompensated rats exhibit 50% lower PKB/Akt activity and severe insulin resistance despite an exercise training-induced increase in total GLUT4 protein (2-fold). Interestingly, insulin receptor tyrosine kinase (Derave et al., 2000) and PI3-kinase (Derave et al., 2000; Kawanaka et al., 2000) activities were similar in

muscle with high or low glycogen. These results suggest that, under conditions of elevated muscle glycogen, insulin-stimulated translocation of GLUT4 is impaired by decreased PKB/Akt activation. How PKB/Akt is inhibited is currently unknown.

Azpiazu et al. (2000) only evaluated insulin-stimulated glucose uptake under conditions of high glycogen in the glycogen synthase transgenic mice. Therefore, it is likely that the lack of increased insulin-stimulated glucose uptake in transgenic compared to wildtype muscle observed may have been due to elevated glycogen levels. It may be possible that increased glycogen synthase activity can influence glucose uptake in muscle when muscle glycogen concentrations of transgenic and wildtype have been reduced. However, this has yet to be demonstrated. To date, no study has assessed the relative importance of exercise and glycogen synthase activity on skeletal muscle insulin-stimulated glucose uptake independent of glycogen levels.

CHAPTER VI: DETAILED METHODS

Experimental Animals and Animal Care

The GSL3 line of transgenic mice used in this study overexpress a constitutively active glycogen synthase [GS(2,3a)], a rabbit skeletal muscle glycogen synthase having Ser to Ala mutations at NH_2 sites 2 and 3a. Transgenic pups were identified by using the polymerase chain reaction to detect chloramphenicol acetyltransferase sequences in tail DNA (donated by Dr. John Lawrence, University of Virginia, Charlottesville, VA). Transgenic expression of the constitutively active glycogen synthase is 10 times higher compared to wildtype muscle, with 5 times greater expression in fast twitch versus slow twitch muscle (Manchester et al., 1996). Mice were individually housed at The University of Texas Animal Resource Center on a 12 h light cycle. Standard rat chow and water were provided ad libitum. Animals were transferred to the laboratory at least 24 h prior to the first experimental procedures and were kept on the same light cycle and provided food and water ad libitum throughout the experiments except where noted below. The University of Texas Animal Care and Use Committee approved all procedures for this study.

Generation of GSL3 Transgenic Mice (from Manchester et al., 1996). CDNA encoding GS(2,3a) was inserted into the *Bst*EII site of the p3300MCKCAT. Just upstream of this *Bst*EII site is the sequence from –3300 to +7 of the mouse creatine kinase gene, which includes elements that direct

expression to both skeletal and cardiac muscle. Immediately adjacent on the downstream side of the *Bst*EII site is a chloramphenicol acetyltransferase reporter followed by a simian virus 40 intron and polyadenylylation site. After digesting p3300MCKCAT with *Bst*EII, the vector was blunt-ended and dephosphorylated. A fragment containing GS(2,3a) cDNA was excised from pCMV-GS(2,3a) by using *Bgl*II and *Xba*I, then blunt-ligated into p3300MCKCAT. Proper orientation of the insert was confirmed by nucleotide sequencing. A fragment containing the 3.3-kb creatine kinase gene sequence, GS(2,3a) cDNA, and simian virus 40 sequences was excised using *Hind*III followed by a partial digest with *Kpn*I, inserted between the *Hind*III and *Kpn*I sites of pSL1180, then excised with *Hind*III and *EcoRv*. The 7.5-kb fragment was purified and injected into the pronuclei of fertilized mouse eggs [(C57BL6 X CBA)F_1 X (C57BL6 X CBA)F_1]. Embryos were implanted into pseudopregnant females and transgenic pups were identified by using the polymerase chain reaction to detect chloramphenicol acetyltransferase sequences in tail DNA. Three founder lines (GSL3, GSL25, and GSL30) were established by mating transgene-positive animals to (C57BL6 X CBA)F_1 mice.

Study 1 Experimental Design. GSL3 transgenic and wildtype mice (n=60) weighing 20-35 g were assigned to either non-exercised or exercised treatment groups. An additional group of wildtype mice (designated SUPER, n=12) was assigned to a glycogen supercompensation group. All mice underwent an intraperitoneal glucose tolerance test (IPGTT) followed approximately 1-2 weeks later by the hindlimb perfusion procedure. Wildtype and transgenic non-exercised

groups (designated WT and TG, respectively) were allowed free access to laboratory chow and water and were otherwise restricted to normal cage activities. The wildtype and transgenic exercised groups (designated WTX and TGX, respectively) underwent two days of glycogen depleting (data not shown) exercise on a motorized treadmill (3 h, 26 m • min^{-1}) with carbohydrate restriction between runs to limit glycogen restoration. Following the first run, mice were given 1 g of laboratory chow with lard provided *ad libitum*. Following the second run mice had free access to lard but did not receive chow. This glycogen depleting exercise/diet protocol was performed prior to both the IPGTT and the hindlimb perfusion procedure allowing glycogen restoration but not supercompensation 24 h following exercise. The wildtype SUPER mice underwent a protocol to raise muscle glycogen levels to above-normal levels. These mice performed an exhaustive run (3 h, 26 m • min^{-1}) followed by 24 h of a high carbohydrate chow/table syrup mix and 15% carbohydrate solution *ad libitum.* This supercompensation procedure was performed prior to both the IPGTT and the hindlimb perfusion. Mice from all groups were fasted (6 h) prior to both the IPGTT and the hindlimb perfusion.

Study 2 Experimental Design. GSL3 transgenic (n=6) and wildtype mice (n=6) weighing 20-35 g were assigned to either non-exercised or exercised treatment groups. All mice in the subsequent four groups were surgically prepared for in situ electrical stimulation and hindlimb perfusion assessment of 2-deoxyglucose uptake. Post stimulation [^{14}C] Glucose incorporation into glycogen was assessed in a different group of wildtype (n = 7) and transgenic (n = 5) mice

weighing 20-35 g. All mice were subjected to a 6 h fast prior to the experimental procedures.

Intraperitoneal Glucose Tolerance Test

Following a 6 h fast, the mice were placed in a restrainer placed on a heating pad to maximize tail blood flow. The tip (2 mm) of the tail was clipped, washed with EDTA (24 mg • ml^{-1} EDTA) and blotted with clean gauze. The mice were given 1 mg • g^{-1} body weight of a 10% glucose solution by intraperitoneal injection. Blood glucose was assessed on a single drop of whole blood prior to and 30, 60, 120, and 150 min following glucose administration. In addition, approximately 0.15 ml of blood was taken prior to, 30 and 60 min following glucose administration. The blood samples were added to tubes containing 24 mg • ml^{-1} EDTA. Following a 10 min centrifugation in a benchtop centrifuge (3,000 g), the plasma was stored at -80ºC until analysis for insulin.

Blood Analysis

Blood glucose was determined with the ONE TOUCH BASIC blood glucose monitoring system from Johnson & Johnson (Milpitas, CA). This system requires a small drop of whole blood onto a reading strip, which is then read by the monitoring unit. Validity of this blood glucose monitoring system was tested against a YSI blood glucose analyzer (Yellow Springs Instrument Company, Yellow Springs, OH). The blood glucose is expressed as mM. Plasma insulin was determined by radioimmunoassay kit (Linco Research, St. Charles, MO) utilizing the double antibody procedure of Morgan and Lazarow (1963). The ^{125}I-labeled

insulin was added to tubes containing plasma samples, standards, and quality controls. Antiserum to rat insulin, was then added. Tubes were incubated overnight at 4ºC during which time the ^{125}I-labeled and unlabeled insulin competed for antibody binding. This antibody-antigen complex was then precipitated by the addition of a carrier and an antibody to the carrier. The bound and free ^{125}I-labeled insulin was separated by centrifugation for 20 min at 3,000 g. Standards were used to construct a standard curve and the samples compared to this curve and expressed as $\mu U \cdot ml^{-1}$.

Hindlimb Perfusion

Mice were anesthetized with an intraperitoneal injection of pentobarbital sodium (6.5 mg • 100 g body weight^{-1}) following a 6 h fast. The surgical technique was similar to that described previously (Brozinick et al., 1996; Ivy et al., 1983). All of the major vessels branching from the abdominal aorta and vena cava except the common iliac artery and iliac vein were ligated and the great vessels cannulated so that perfusate could flow into the iliac artery, through the capillary bed of the hindlimbs and out of the abdominal section of the inferior vena cava. Both hindlimbs were perfused during the equilibrium (10 min) and tracing (20 min) periods. Anesthetized mice were kept warm by performing the surgery on a heating pad. The tail was ligated with string and a midline incision made through the skin and abdominal wall from the xiphoid process to the pubic symphysis. The skin was retracted and the superficial epigastric arteries leading to the skin ligated with suture. The inferior vena cava and descending aorta was exposed by lifting the stomach, spleen, pancreas, and intestinal tract from the

abdominal cavity, wrapping these organs in parafilm and placing them along side the animal. The hypogastric arteries on the inside of the abdominal wall were ligated. Next the testes were pulled up into the abdominal cavity. Suture was then used to ligate the seminiferous vesicles, testes, and bladder collectively. For female mice, suture was used to ligate the fallopian tubes, ovaries, and bladder. These organs were then removed. The descending colon was excised after ligation just inferior to the transverse colon and just superior to the rectum. Adipose tissue and fascia remaining in the abdominal cavity anterior to the aorta and vena cava was removed by gently spreading with gauze dampened in Krebs-Henseleit buffer (KHB). The iliolumbar arteries and veins and pubic-epigastric trunk were also ligated.

Once ligation of the major vessels is completed, loose ligatures were placed around the aorta and vena cava just inferior to the renal vessels and the vena cava just superior to the iliac bifurcation. Another loose ligature was placed around the left iliac artery and vein. Next, heparin (100 U) was injected into the inferior vena cava just inferior to the diaphragm.

Following the heparin injection, the descending aorta and inferior vena cava were cannulated. First, the ligature around the descending aorta located proximal to the cannula insertion and just inferior to the renal bifurcation was ligated. The aorta was cannulated and the catheter inserted until the tip was just superior to the bifuracation of the iliac arteries. The ligature around the inferior vena cava at the level of the renal vessels was tied and KHB slowly infused, causing the vessel to distend. The vena cava was then cannulated and the venous

catheter inserted past the loose distal ligature and superior to the ligated iliolumbar vessels and secured into place. The hindlimbs were then flushed by slow infusion of 8 ml KHB through the arterial catheter to remove most of the mouse's blood and prevent clotting. During the infusion of KHB, the mouse was euthanized with an intracardiac injection of pentobarbital sodium. After the infusion, the catheters were placed in line with the hindlimb perfusion apparatus.

Hindlimb Perfusion Apparatus

The perfusion apparatus consisted of an arterial reservoir, pump, lung, water jacket, bubble trap and effluent reservoir. A peristalic pump pulled perfusate out of the arterial reservoir consisting of a beaker set upon a magnetic stir plate. The perfusate was pumped first through a "lung" consisting of 3 meters of gas permeable Silastic tubing (Dow Corning, Midland, MI) contained within a chamber receiving a flow of 95% O_2 and 5% CO_2 mixed gas. The perfusate then passed through a water jacket heated to 37ºC, a bubble trap, and into the aorta of the mouse. Finally, the venous effluent drips into the effluent reservoir. The flow rate of the peristalic pump throughout the perfusion period was 1.8 ml • min^{-1}. A manometer connected to the tubing just prior to the bubble trap measured arterial pressure.

Perfusate Preparation

All perfusates consisted of 6% bovine serum albumin (BSA) in KHB. Initially, a 20% BSA solution was made in KHB. This solution was dialyzed for 48 hours against 15 L KHB and filtered through a glass fiber filter (# 61631,

Gelman) and then a 0.45 µm filter (GN-6, Gelman). The BSA solution was then diluted to 8-10% with KHB as measured by a refractometer. The appropriate volume of this solution was added to the perfusate to bring the BSA concentration to 6%. The pH of the perfusion was adjusted to 7.5 with 10 N NaOH. During the perfusion the pH of the perfusate falls to 7.4 due to the dissolving of CO_2 introduced from the gassing apparatus explained above. This perfusate was separated into two aliquots for the equilibrium and tracer periods, respectively. During the equilibrium period, the perfusate also contained 0.2 mM pyruvate as a cellular energy source. During the tracer period, the perfusate contained 6 mM [1,2-^{3}H] 2-deoxyglucose (7.5 µCi • mmol^{-1}), 2 mM [U-^{14}C] mannitol (60 µCi • mmol^{-1}) and the appropriate insulin (Humulin R-100, Eli Lilly, Indianapolis, IN) concentration. The perfusion insulin concentrations, 0.2 mU • ml^{-1} or 10 mU • ml^{-1}, represent submaximal and maximally effective hormone concentrations, respectively.

Muscle Contraction Procedure

Following a 6 h fast, mice were anesthetized with pentobarbitol sodium (6.5 mg • 100 g body weight^{-1}). The skin from the right hindlimb was reflected and a section of the calcaneous, with the Achilles tendon still attached, clipped from the foot. The triceps surae muscle group was retracted from the tibia and the distal tendon (detached from the bony insertion) of the muscle group was attached to an isometric force transducer. The sciatic nerve was isolated and placed in-line, via hook electrode, with a Grass Stimulator (Grass Instruments, Quincy, MA). Both the right hindlimb and the mouse torso were immobilized in a specially

designed cradle and the muscles of the triceps surae group adjusted to achieve maximum twitch tension. Peak twitch (Pt) and peak tetanic (Po) tensions were assessed (Series 300 B Lever System, Aurora Scientific Incorporated, Ontario, Canada) with supramaximal stimulation strength at the optimal length for the muscle contracting at 90º to the knee joint.

The goal of this stimulation procedure was to reduce muscle glycogen prior to the assessment of 2-deoxyglucose uptake. Therefore, a modified version of the protocol described by Johannsson et al. (1996a & 1996b) and Roy et al. (1997) was employed. This protocol was chosen because: 1) it is a 30 min protocol, allowing time for stimulation prep, stimulation, and perfusion prep while the animal remains sedated but alive; 2) produces 90-100 µmol/g reduction in gastrocnemius glycogen (Roy et al., 1997); 3) is designed to be performed in conjunction with a hindlimb perfusion; 4) has demonstrated isometric force decreases of 65% of initial force after 2 min and 50% of initial force after 10 min (Johannsson et al., 1996a); 5) because the protocol's surgery prep is very similar to one recently performed (Lee, S. and R. Farrar, personal communication); and 6) isometric force can be assessed over the entire 30 min protocol and provide an index of fatigue. The sciatic nerve was stimulated via hook electrode. Peak torque was assessed with supramaximal stimulation strength at the optimal length for the muscle. The muscle was stimulated for 30 min using supramaximal (8 V) trains composed of 1 ms square-wave pulses firing at 100 Hz. Trains of 200 ms were delivered at a rate of 30 per min (1 per 2 sec). Tension was assessed throughout the stimulation period and fatigue following 30 min stimulation period expressed

as percent of Po. Gastrocnemius muscle cross sectional area was estimated by dividing wet weight by the measured optimal muscle length (cm) multiplied by the fiber to muscle length ratio for rodent gastrocnemius (0.4) multiplied by mammalian muscle specific gravity (1.06 g/cm^3). Specific force (N/cm^2) was then estimated by dividing muscle tetanic tension (N) by the estimated cross-sectional area (cm^2) (Brooks and Faulkner, 1988). Following the stimulation period, the mice were prepared for the hindlimb perfusion procedure for measurement of insulin-stimulated [^{3}H] 2-deoxyglucose uptake or determination of [^{14}C] glucose incorporation into glycogen using a submaximal insulin concentration (0.2 mU • ml^{-1}). Following the perfusion, stimulated and non-stimulated gastrocnemius muscles were surgically removed, blotted dry and freeze clamped with Wollenberg tongs cooled in liquid nitrogen. Tissue was stored at -80ºC until subsequent analysis.

Measurement of Glucose Uptake

Relative changes in glucose uptake were estimated by determining the rate of incorporation of [^{3}H] 2-deoxyglucose tracer into muscle tissue. 2-Deoxyglucose is a non-metabolized glucose analog that has similar rates of removal from blood into muscle tissue as glucose under a physiologic range of insulin concentrations. The amount of tracer contained within the extracellular space was determined by the amount of [^{14}C] mannitol retained in the tissue. Mannitol is a sugar that is not transported by glucose transporters and therefore occupies only the extracelluar space.

2-Deoxyglucose Uptake Assay Procedure

Freeze-clamped mixed (red and white portions) gastrocnemius muscles from the perfused hindlimbs were sectioned and weighed frozen. A 60-100 mg piece of muscle was dissolved in 1 ml 1 N KOH by incubating for 15 min at 65ºC, mixed, and incubated an additional 5 min at 65ºC. An equal volume of 1 N HCl was added to the digested samples, mixed, and aliquots of the neutralized samples counted for [^{3}H] and [^{14}C] DPM (Beckman LS 6000SC). Muscle 2-deoxyglucose uptake was calculated from the specific activity of the original perfusate after subtracting out the appropriate volume for extracellular space as determined from radiolabelled mannitol in the muscle sample. The mixed gastrocnemius was used because it exhibits a high transgene expression but contains similar GLUT4 content in control and GSL3 transgenic animals (Manchester et al., 1996).

[^{14}C]-Glucose Incorporation into Glycogen

[^{14}C]-Glucose incorporation into glycogen was assessed as previously described (Willems et al., 1991). The perfusion surgery and preparation was essentially the same as described for 2-deoxyglucose uptake until the final perfusion period. Briefly, the hindlimbs were perfused with 6 mM glucose (0.15 µCi • ml^{-1} D-[^{14}C(U)]glucose) with a submaximal insulin concentration (0.2 mU • ml^{-1}). The [^{14}C]glucose was added to determine the rates of glucose incorporation into glycogen. Following the perfusion and dissection (described above) gastrocnemius muscles were weighed frozen, placed in screw-top test tubes containing 1 ml of 30% KOH saturated with Na_2SO_4, and digested by incubating the tubes for 30 min at 100ºC. After the incubation, the tubes were cooled to room

temperature and the glycogen precipitated overnight at 4ºC after the addition of 1:2 volumes of 95% ethanol. The glycogen was pelleted by centrifugation and resuspended in distilled H_2O. An aliquot of this resuspension was transferred to a scintillation vial containing 5 ml of scintillation fluid, and radioactivity determined (Beckman LS 6000SC).

Measurement of Muscle Glycogen

Muscle glycogen concentration was determined by complete enzymatic degradation with amyloglucosidase (Boehringer Mannheim, Indianapolis, IN) by a modified method of Passonneau and Lauderdale (1974). A 100 µl aliquot of neutralized homogenate used for glucose uptake measurements was added to 250 µl of 0.3 M sodium acetate, pH 4.8 and vortexed. Then 250 µl of 0.3 M sodium acetate, pH 4.8, containing 10 mg • ml^{-1} amyloglucosidase was added and vortexed. Following an overnight incubation, 25 µl of 1 N NaOH was added and vortexed. Duplicate 150 µl aliquots were placed in test tubes and subjected to the Trinder reaction (Sigma). Tubes containing 0, 50, 100, and 200 mg% glucose and 0.3 M sodium acetate were used as standards. One milliliter of Trinder reagent was added to each tube, mixed gently and incubated for 18 min at room temperature and then read on a Beckman DU 6 spectrophotometer at an absorbance of 505 nm. A linear interpolation against the standard was used to determine the concentration of glucose units in the original sample. The muscle glycogen concentration was expressed as µmole glucose • g $muscle^{-1}$.

Measurement of Glycogen Synthase Activity

Glycogen synthase was measured by direct incorporation of labeled substrate ([U-^{14}C] UDP-1-glucose) into glycogen with several concentrations of G-6-P (0.01, 0.5, 1.5, and 25 mM) as previously described (Sherman et al., 1988). A 30 to 70 mg piece of muscle was weighed frozen and homogenized at –20ºC in 4 volumes of a buffer containing 25 mM KF, 20 mM EDTA and 60% glycerol and then diluted with 25 mM KF, 20 mM EDTA and centrifuged at 8,000 g for 15 min at 4ºC. The supernatant was transferred to a test tube and further diluted 2-fold with buffer containing 100 mM MOPS and 20 mM EDTA (pH 6.9) while kept on ice. Reaction cocktail was thawed and 0.2 µCi of [U-^{14}C] UDP-1-glucose • ml^{-1} added. The radioactive cocktail was divided into five aliqouts. One aliquot contained 150 mM G-6-P. Two 1:9, followed by one 1:1, serial dilutions were performed from a 150 mM aliquot to yield G-6-P concentrations of 0.75, 1.5, 15 mM. The final assay G-6-P concentrations were 0.01, 0.5, 1.5, and 25 mM. Relative activities were expressed as the I/(I + D) ratio, the G-6-P-independent activity divided by the activity with maximal G-6-P concentration. In addition, fractional velocity of submaximal (0.5 and 1.5 mM G-6-P) to that of maximal (25 mM) G-6-P were measured.

Insulin Signaling Proteins

Muscles were homogenized in ice-cold buffer containing 50 mM HEPES (pH 7.4), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 1.0 mM EDTA, 10 mM $Na_4P_2O_7$, 10 mM NaF, 2.0 mM Na_3VO_4, aprotinin (10 µg • ml^{-1}), leupeptin (10 µg • ml^{-1}), pepstatin (0.5 µg • ml^{-1}), 1% Igepal and 2 mM

phenylmethylsulfonyl fluoride. Homogentates were incubated with end-over-end rotation at 4ºC for 60 min and then centrifuged at 200,000 *g* for 50 min at 4ºC.

IRS-1-associtated PI3-kinase was immunoprecipitated using a 2 µg • µl^{1} protein sample as previously described (Christ et al., 2001) using an anti-IRS-1 antibody (2 µg, Upstate #06-248) for 2 h at 4ºC, followed by protein A-Sepharose (4ºC, overnight; Sigma, St. Louis, MO). The immunoprecipitates were successively washed and PI3-kinase activities measured immediately on immunoprecipitates by *in vitro* phosphorylation of phosphatidylinositol. Sonicated phosphatidylinositol was added to each sample and the PI3-kinase reaction started by addition of 10 µl of 50 mM $MgCl_2$250 µM [γ-^{32}P] ATP (0.5 µCi • ml^{-1}, Amersham) in a buffer consisting of 20 mM HEPES (pH 7.4), 0.4 mM EGTA, and 0.4 mM Na_2PO_4 and stopped by addition of 6 M HCl. Lipids were extracted by chloroform-methanol (1:1) and applied to a silica gel TLC plate (Silica gel 60, Whatman, Hillsboro, OR). The plates were developed (60 min) in running solvent ($CHCl_3$:MeOH:H_2O:NH_4OH, 60:47:11.3:2). Radioactivity of TLC spots for standards and samples was quantified via scintillation counting for ^{32}P (Beckman LS 6000SC).

Three isoforms of PKB/Akt have been identified. The primary insulin-stimulated isoform in rodent muscle is PKB/Akt 1 (Walker et al., 1998). We have assessed phosphorylated PKB/Akt as an index for insulin-stimulated activation. For quantification of phosphorylated PKB/Akt, aliquots of a 200,000 *g* supernatant were treated with 2x Laemmli sample buffer containing 100 mM dithiothreitol and boiled for 5 min. Samples and insulin-stimulated mouse

gastrocnemius standards (80 μg protein) were subjected to SDS-PAGE (10% resolving gel) and transferred to nitrocellulose membranes. The membranes were blocked in 5% nonfat dry milk in TBS containing 0.1% Tween 10 (pH 7.5) overnight. PKB/Akt is activated by phosphorylation of Thr^{308} in the core activation loop and Ser^{473} in the carboxy terminus of the enzyme (Alessi et al., 1996). Therefore, the membranes were rinsed in 0.1% Tween 10 and incubated with sheep antiphospho-PKB/Akt (Ser^{473}) antibody (Upstate Biotechnology Inc.) for 4 h. The membranes were rinsed in 0.1% Tween 10 and incubated with horseradish peroxidase-conjugated rabbit anti-sheep IgG (Jackson ImmunoResearch) for 60 min. Antibody-bound protein was visualized by ECL (Amersham). The intensity of the bands corresponding to Ser^{473}-phosphorylated PKB/Akt was assessed using a computer scanner and Adobe Photoshop 9.0 software and expressed as a percentage of insulin-stimulated mouse gastrocnemius standard.

Total PKB/Akt protein content was assessed on nitrocellulose membranes used for phosphorylated PKB/Akt. Membranes were stripped of antibodies by incubation with stripping buffer containing 100 mM 2-mercaptoethanol, 2% sodium dodecyl sulfate, 62.5 mM Tris HCl (pH 6.7) for 30 min. Membranes were then washed three times for 15 min in TTBS buffer. Membranes were blocked as described above overnight washed three more times for 15 min in TTBS and incubated with sheep anti-Akt/PKBα (1:1000 vol/vol) (Upstate for 4 h with gentle agitation. Following three more TTBS washes, antibody-bound protein was visualized using ECL (Amersham) and total PKB/Akt was expressed relative to

the insulin-stimulated mouse standard. The intensity of the bands corresponding to total PKB/Akt was assessed using a computer scanner and Adobe Photoshop 9.0 software and expressed as a percentage of insulin-stimulated mouse gastrocnemius standard.

Western Blotting Analysis for GLUT4 Protein

Muscle homogenates (1:20) in HES buffer were denatured 1:1 in 2x Laemmli buffer (125 mM Tris, 20% glycerol, 2% SDS, 0.008% bromopherol blue, pH 6.8). Muscle sample protein content was determined as described by Bradford (1976). A 75 µg protein aliquot of each sample or the standard was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12.5% polyacrylamide resolving gel for 55 min at 10 mA (for two units). A mouse heart homogenate (16 µl) was run on each gel as a standard. A Miniprotean II dual slab cell (BioRad, Richmond, CA) was used to perform the electrophoresis. Samples were transferred from the resolving gels onto polyvinylidene diflouride (PVDF) sheets (BioRad, Richmond, CA). The transfer procedure was performed for 12 min at 30 mM. The PVDF sheets were stored in water overnight at 4ºC. The transferred PVDF membranes was blocked for 1 h by incubation in 5% nonfat dry milk (Carnation, Los Angeles, CA) in TTBS (0.06% Tween-20, 20 mM Tris, 500 mM NaCl, pH 7.5) at room temperature. Following a rinse using TTBS, the membranes were incubated in GLUT4 rabbit anti-rat antibody (diluted 1:500; donated by Dr. Samuel Cushman, NIH, Bethesda, MD). After two additional 5 min rinses in TTBS, the membranes were incubated with horseradish peroxidase (HRP)-conjugated donkey anti-rabbit antibody

(Amersham, Arlington Heights, IL) diluted 1:5000 for one hour. The membranes were then washed in sacosyl buffer (50 mM Tris Base, 1 M NaCl, O.4% w/v N-laurylsarcosine, 5 mM EDTA, pH 7.5) three times to decrease non-specific binding. After the blotting procedure, the membranes were placed in a small container with 6 ml of mixed detection reagent (equal volume of no. 1 and 2 from Amersham, Arlington Heights, IL) for 1 min. The membranes were removed and sealed in plastic wrap for protection. Sealed membranes were placed into x-ray film cassettes that contain an intensifying screen (Quanta III, Dupont Cronex, Wilmington, DE). Hyperfilm (Amersham) was loaded onto the cassette and exposed for 90 sec. GLUT4 bands was visualized by using a computer scanner and Adobe Photoshop 9.0 software and expressed as a percentage of heart standard.

Measurement of Hexokinase Activity

Frozen muscle was homogenized (1:20) in HES buffer containing 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffer, 1 mM EDTA, and 250 mM sucrose (pH 7.4). For hexokinase determination, muscle homogenates were centrifuged in a benchtop centrifuge at 3,000 g for 15 min. Hexokinase activity was measured on the supernatant using the spectrophotometric determination of glucose-6-phosphate as described by Uyeda and Racker (1965).

CHAPTER VII: SUMMARY OF FINDINGS AND FUTURE DIRECTIONS

Summary of Findings

By utilizing the GSL3 transgenic mouse, which overexpresses glycogen synthase, we have investigated the effects of glycogen synthase on post-exercise muscle glucose metabolism independent of muscle glycogen concentration. In Study 1, we determined the effects of exercise on whole body glucose tolerance and insulin-stimulated skeletal muscle glucose uptake and insulin signaling both pre- and post-exercise independent of muscle glycogen concentration. Study 2 investigated the effects of elevated glycogen synthase activity on insulin-stimulated muscle glucose uptake and glycogen synthesis immediately following a lowering of initial glycogen levels by *in situ* electrical stimulation of muscle. It was hypothesized that transgenic muscle would exhibit a greater post-exercise insulin-stimulated muscle glucose uptake independent of muscle glycogen level. In other words, because of the increased activity of glycogen synthase in the muscle of these mice, enhanced post-exercise insulin action will be maintained regardless of glycogen concentration.

In Study 1, we provided evidence that muscle glycogen concentration, *per se*, does not affect post-exercise insulin action *in vivo* or in muscle using an *in situ* preparation. Following exercise, GSL3 transgenic muscle exhibits greater insulin action and insulin-stimulated muscle glucose uptake independent of muscle glycogen content relative to wildtype muscle. The increase in insulin-stimulated

muscle glucose uptake following exercise in GSL3 transgenic muscle could not be accounted for by increases in GLUT4 protein concentration or hexokinase activity. Glycogen concentration appears to be inversely-related to post-exercise muscle insulin signaling and insulin-stimulated glucose uptake in wildtype muscle when elevated above normal. However, our insulin signaling results support the possibility that the normal mechanisms responsible for the inverse relationship between muscle glycogen concentration and glucose uptake may be absent in GSL3 transgenic muscle.

Study 2 demonstrated that increased glycogen synthase activity prevents glycogen from compromising post-contraction insulin-stimulated muscle glucose uptake and glucose incorporation into glycogen. Electrical stimulation of muscle *in situ* via the sciatic nerve reduced muscle glycogen concentration by 49% in wildtype and 27% in GSL3 transgenic. However, GSL3 transgenic muscle retained a 9.6-fold greater muscle glycogen concentration than wildtype. While GSL3 transgenic and wildtype muscle demonstrated similar post-contraction increases in insulin-stimulated glucose uptake, insulin-stimulated incorporation of [^{14}C] glucose into glycogen was approximately 150% greater in non-stimulated and electrically stimulated transgenic versus the respective wildtype treatments. Insulin-stimulated glycogen synthase responsiveness and G-6-P sensitivity, as well as PKB/Akt Ser^{473} phosphorylation were greater in transgenic as compared to wildtype post-contraction. Contractions increased insulin-stimulated PKB/Akt phosphorylation in wildtype but not in transgenic muscle.

In summary, glycogen concentration appears to influence post-exercise muscle insulin signaling and insulin-stimulated glucose uptake in wildtype muscle when elevated above normal. However, our insulin signaling results support the possibility that the normal mechanisms responsible for the inverse relationship between muscle glycogen concentration and glucose uptake is absent in GSL3 transgenic muscle. This may represent a putative, yet unknown, association between the activation states of glycogen synthase and PKB/Akt, both of which are normally inhibited when muscle glycogen has been elevated above normal. In addition, overexpression of glycogen synthase may facilitate a repartitioning of intracellular glucose intermediates towards glycogen synthesis as opposed to the glycolytic, hexosamine, and oxidative pathways. Using the GSL3 transgenic mouse model, we have provided evidence that muscle glycogen concentration, *per se*, does not affect post-exercise insulin action *in vivo* or in muscle using an *in situ* preparation.

Future Directions

Little is known about the molecular mechanisms that convert skeletal muscle contractile activity into biochemical and gene regulatory responses. The convergence and integration of multiple signals in response to contractile activity play an important role in controlling metabolism. PKB/Akt appears to play a pivotal role in both insulin regulation of glucose transport and glycogen synthase activity. However, a number of other important signaling proteins need to be assessed using our experimental model. Two prominent signaling proteins, which

we have implicated as possibly influencing our results, are GSK-3 and IRS-1. Recent research suggests that insulin activates muscle glycogen synthase in part by decreasing the activity of GSK-3 (Cross et al., 1997; Ueki et al., 1998). In skeletal muscle, this GSK-3 inactivation may be mediated by insulin-stimulated PKB/Akt activity (Cross et al., 1995). GSK-3, in turn, has been implicated in feedback inhibition of IRS-1 facilitated PI3-kinase activity (Eldar-Finkelman and Krebs, 1997; Summers et al., 1999). Therefore, by assessing the interaction of these proteins, we could better determine where the major point of influence by glycogen is in the insulin-mediated glucose uptake pathway.

Other important questions that need to be addressed concern the metabolism of the GSL3 transgenic mouse during exercise. Although we did not observe a difference in running mechanics or time to exhaustion during Study 1, we do not know if the fuel usage during exercise is similar to that in the wildtype mice. For instance, with the overexpression of a constitutively active glycogen synthase, GSL3 transgenic muscle glycogen may simply be recycled into glycogen as soon as it is hydrolyzed. In this scenario, the GSL3 transgenic mice may not have been able to use muscle glycogen as a primary fuel source during exercise. If this indeed proved to be the case, then blood glucose, free fatty acid and intramuscular triglyceride usage should be measured. In addition, we did not measure lactate in the muscle or lactic acid in the blood following the exercise bout. If GSL3 transgenic mice were, in fact, able to utilize glycogen as a fuel source we would expect lactate production to be greater than in wildtype mice.

Transgenic overexpression provides a means to selectively alter the activities of enzymes within a metabolic pathway. One must keep in mind that the effects observed with long-term overexpression of glycogen synthase are not necessarily representative of the acute activation of glycogen synthesis by insulin. In addition, development of compensatory responses to the transgenic expression must be considered. An example is the increase in phosphorylase observed in GSL3 muscle by Manchester et al. (1996). Conversely, overexpression of phosphorylase was accompanied by increased glycogen synthase in cultured human skeletal muscle cells (Baquè, et al., 1996). Therefore, another future direction would be to further investigate the role of glycogen concentration on insulin action under physiological conditions were muscle glycogen has been raised to above normal levels.

APPENDIX A: SOLUTIONS

The following is an alphabetical listing of solutions used throughout the studies presented. The final volume of the prepared solution follows the name of each solution. Contents of the solutions are listed followed by instructions for their preparation. Final concentration is the concentration of the listed reagent after preparation of the solution. Amount needed is the mass or volume of reagent used. FW or Stock Con. is the formula weight or stock concentration of the reagent used to prepare the solution.

ACETIC ACID 0.3M (100 ML)

Final Concentration	Amount Needed	FW or Stock Con.
0.3 M	1.72 ml	17.4 M Stock

ATP 50MM (5ML)

Final Concentration	Amount Needed	FW or Stock Con.
50mM	0.1378 g	FW 551.1

ATP was added to 5ml dH2O stirring on a magnetic and pH to 7.0

^{32}P-ATP SOLUTION (150µL)

Final Concentration	Amount Needed
10mM ATP	3µl

4XHEPES buffer	37.5µl
0.4M $MgCl_2$-$6H_20$	25µl
32P-ATP	24µl

ß-GLYCEROPHOSPHATE 1.0M (45ML)

Final Concentration	Amount Needed	FW or Stock Con.
1.0M	9.72g	FW 216.0

ß-glycerophosphate was added to 35ml dH2O stirring on a magnetic stir plate, and then brought to volume with dH2O

CALCIUM CHLORIDE 0.5M (45ML)

Final Concentration	Amount Needed	FW or Stock Con.
0.5M	2.498g	FW 110.0

Calcium chloride was added to 35ml dH2O stirring on a magnetic stir plate, and then brought to volume with dH2O

COOSMASSIE BLUE SOLUTION (25ML)

Final Concentration	Amount Needed	FW or Stock Con.
0.1% brilliant blue	0.05g	FW 826.0
40% methanol	10ml	99.8% methanol stock
10% acetic acid	5ml	99.7% acetic acid stock

The following agents was added to 20ml dH2O stirring on a magnetic stir plate, and then brought to volume with dH2O

CUPRIC SULFATE (25ML)

Final Concentration	Amount Needed	FW or Stock Con.
0.1% cupric sulfate	0.25g	FW 249.6

Cupric sulfate was added to 20ml dH2O stirring on a magnetic stir plate, and then brought to volume with dH2O

EDTA 80MM (35 ML)

Final Concentration	Amount Needed	FW or Stock Con.
80mM	1.191g	FW 372.2

EDTA was added to 30ml dH2O stirring on a magnetic plate, and then brought to volume with dH2O and pH at 8.0

DITHIOTHREITOL 1.0M (DTT) (2ML)

Final Concentration	Amount Needed	FW or Stock Con.
1.0M	0.308g	FW 154.25

Dithiothreitol was added to 2ml H2O.

GLUCOSE (250ML)

Final Concentration	Amount Needed	FW or Stock Con.

1M	45.05g	FW 180.2

D-Glucose was added to 200ml dH2O stirring on a magnetic plate, and then brought to volume with dH2O

GLYCINE HYDRAZINE BUFFER pH 9.2 (1L)

Final Concentration	Amount Needed	FW or Stock Con.
0.33 M Glycine	25.02 ml	FW 75.07
0.27 M Hydrazine	23.98 ml	54% hydrazine pH 9.2

Reagents were added in the order listed to 800 ml dH2O stirring on a magnetic stir plate, titrated with 10N HCl and 10 N NaOH and then brought to volume with dH2O.

GLYCOGEN SYNTHASE HOMOGENATE SUPERNATANT DILUTION BUFFER (50 ML)

Final Concentration	Amount Needed	FW or Stock Con.
20 mM EDTA	0.293 g	FW 292.2
100 mM MOPS	1.05 g	FW 209.3

Reagents were added in order listed to 40 ml dH2O stirring on a magnetic stir plate, titrated with 5 N NaOH and brought to volume with dH2O. pH 6.9.

GLYCOGEN SYNTHASE HOMOGENIZING BUFFER I (50 ML)

Final Concentration	Amount Needed	FW or Stock Con.

50 mM KFl	0.1453 g	FW 58.1
20 mM EDTA	0.292 g	FW 292.2
60% glycerol	30 ml	99% Stock

Reagents were added in the order listed to 15 ml dH2O stirring on a magnetic stir plate, titrated with 1 N NaOH and then brought to volume with dH2O. pH 7.0.

GLYCOGEN SYNTHASE HOMOGENIZING BUFFER II (50 ML)

Final Concentration	Amount Needed	FW or Stock Con.
50 mM KFl	0.1453 g	FW 58.1
20 mM EDTA	0.292 g	FW 292.2

Reagents were added in the order listed to 15 ml dH2O stirring on a magnetic stir plate, titrated with 1 N NaOH and then brought to volume with dH2O. pH 7.0.

HEPES 1.0M (45ML)

Final Concentration	Amount Needed	FW or Stock Con.
1M HEPES	10.725 g	FW 238.8

HEPES was added to 35ml dH2O stirring on a magnetic plate, and then brought to volume with dH2O

4XHEPES pH 7.6 (100ML)

Final Concentration	Amount Needed	FW or Stock Con.
200mM HEPES	4.77g	FW 238.8

40uM EGTA	0.15g	FW 372.2
3mM NaH_2PO_4	0.048g	FW 142.0

HEPES was added to 70ml dH2O stirring on a magnetic plate, pH to 7.6 and then brought to volume with dH2O

HOMOGENIZATION BUFFER PI3 KINASE (20ML)

Final Concentration	Amount Needed	FW or Stock Con.
50mM HEPES	1ml	1.0M HEPES (pH 7.6)
150mM NaCl	600µl	5.0N NaCl
20mM Na-pyrophosphate	4ml	100mMNapyrophosphate
20mMB-glycerophosphate	400µl	1.0M glycerophosphate
10mM Na F	400µl	0.5N NaF
2mMNa-OrthoVanadate	400µl	100mMNa-OrthoVanadate
2mM EDTA	500µl	80mM EDTA
1%IGEPAL	2ml	10% IGEPAL
10% Glycerol	2ml	100% Glycerol
2mM Phyenylmethylsufonyl	400µl	100mM Phenylmethylsufonyl fluoride
1mM $MgCl_2$	50µl	0.4M $MgCl_2$
1mM $CaCl_2$	40µl	0.5M $CaCl_2$
10ug/ml Aprotinin	200µl	1mg/ml

10ug/ml Leupeptin	40µl	5mg/ml Leupeptin

Add solutions in order while stirring on a stir plate. Once all ingredients have been added, bring to volume with dH_2O

HYDROCHLORIC ACID 1N (1L)

Final Concentration	Amount Needed	FW or Stock Con.
1N HCL	86ml	11.6 N ACS Reagent

Concentrated hydrocholric acid was slowly added to 800ml dH2O stirring on a magnetic stir plate and then brought to volume with dH2O.

HYDROCHLORIC ACID 5N (100ML)

Final Concentration	Amount Needed	FW or Stock Con.
5N HCL	34.5ml	11.6 N ACS Reagent

Concentrated hydrocholric acid was slowly added to 65.5ml dH2O stirring on a magnetic stir plate and then brought to volume with dH2O

10% IGEPAL (100ML)

Final Concentration	Amount Needed	FW or Stock Con.
10%	10ml	100% IGEPAL stock

10ml IGEPAL was added to 90ml dH2O stirring on a magnetic stir

KREBS-HENSELEIT BUFFER (3L)

Final Concentration	Amount Needed	FW or Stock Con.
1X Krebs-Henseleit	300ml	10X KHB Stock 1
1x Krebs-Henseleit	300ml	10X KHB Stock 2

Krebs-Henseleit Stock 1and 2 was added to 1L dH2O stirring on a magnetic stir plate and the solution was gassed for 45 min with 95%O2, 5% CO2. Then the solution was brought to volume.

KREBS-HENSELEIT DIALYSIS BUFFER (15L)

Final Concentration	Amount Needed	FW or Stock Con.
116 mM NaCl	101.53 g	FW 58.44
4.6 mM KCl	5.143 g	FW 74.56
2.5 mM CaCl2•2H2O	5.512 g	FW 147.0
1.16 mM KH2PO4	2.367 g	FW 136.1
1.16 mM MgSO4•7H2O	4.289 g	FW 246.48

Reagents were added in order listed to 12.5 L dH2O stirring on a magnetic stir plate. Then 2.5 L of well-gassed NaHCO3 (sodium bicarbonate buffer) was added.

KREBS-HENSELEIT STOCK 1 10X KHB (2L)

Final Concentration	Amount Needed	FW or Stock Con.
1.16M NaCl	135.58g	FW 58.44
46mM KCl	6.858g	FW 74.56

253.0 mM NaHCO3	42.50g	FW 84.01
11.6 mM KH2PO4	3.156g	FW 136.1

Reagents were added in the order listed to 1600ml dH2O stirring on a magnetic plate, and then brought to volume with dH2O

KREBS-HENSLEIT BUFFER STOCK 2 10 X KHB (2L)

Final Concentration	Amount Needed	FW or Stock Con.
25Mm CaCl2. 2H2O	7.350g	FW 147.0
11.6mM MgSO4.7H2O	5.718g	FW 246.48

Reagents were added in the order listed to 1600ml dH2O stirring on a magnetic stir plate and the brought to volume with dH20.

LITHIUM CHLORIDE 2.0M (50ML)

Final Concentration	Amount Needed	FW or Stock Con.
2.0M	4.239g	FW 42.39

Lithium chloride was added to 35ml dH2O stirring on a magnetic stir plate, and then brought to volume with dH2O

MAGNESIUM CHLORIDE 0.4M (45ML)

Final Concentration	Amount Needed	FW or Stock Con.
0.4M	3.659g	FW 202.3

Magnesium chloride was added to 35ml dH2O stirring on a magnetic stir plate, and then brought to volume with dH2O

MANNITOL (250ML)

Final Concentration	Amount Needed	FW or Stock Con.
500mM	22.78g	FW 182.2

Mannitol was added to 200ml dH2O stirring on a magnetic stir plate, and the brought to volume with dH2O.

PBS (500ML)

Final Concentration	Amount Needed	FW or Stock Con.
136mM NaCL	4g	FW 58.44
2mM KCl	0.1g	FW 74.5
1mM KH_2PO_4	0.12g	FW 136.0
9mM Na_2HPO_4	7g	FW 142

Reagents were added to 400ml dH2O stirring on a magnetic stir plate, and the brought to volume with dH2O, then pH at 7.4.

PERCHLORIC ACID 6% (100ML)

Final Concentration	Amount Needed	FW or Stock Con.
6% PCA	8.57ml	70% PCA reagent

Concentrated perchloric acid was slowly added to 80ml dH2O stirring on a magnetic stir plate, then brought to volume with dH2O.

PHENOLMETHYLSULFONYLFLUORIDE 100MM (40ML)

Final Concentration	Amount Needed	FW or Stock Con.
100mM	0.6968 g	FW 174.19

Phenolmethylsulfonylfluoride was added to 30ml 100% ethanol stirring on a magnetic stir plate, and then brought to volume with ethanol.

POTASSIUM BICARBONATE 30% (100ML)

Final Concentration	Amount Needed	FW or Stock Con.
30% KHCO3	30g	FW 100.1

Potassium bicarbonate as added to 70ml dH2O stirring on a magnetic stir plate, gently heated and then brought to volume with dH2O. This is saturated solution at room temperature.

POTASSIUM HYDROXIDE 1N (1L)

Final Concentration	Amount Needed	FW or Stock Con.
1N KOH	56.1g	FW 56.11

Potassium hydroxide pellets were added to 800ml dH2O stirring on a magnetic stir plate, and then brought to volume with dH2O.

PTA (500ML)

Final Concentration	Amount Needed	FW or Stock Con.
Tween 20	0.1g	Stock
SDS	0.2g	FW 288.4
NaN_3	0.04g	FW 65.0

Reagents were added to 400ml dH2O stirring on a magnetic stir plate, and the brought to volume with dH2O, then pH at 7.4 and filtered sterilized.

PYRUVATE (250ML)

Final Concentration	Amount Needed	FW or Stock Con.
750mM Na-pyruvate	20.63g	FW 110

Sodium pyruvate was added to 200ml dH2O stirring on a magnetic stir plate, and then brought to volume with dH2O.

SODIUM ACETATE (1L)

Final Concentration	Amount Needed	FW or Stock Con.
0.3M Sodium Acetate	40.83g	FW 136.1

Sodium acetate was added to 800 ml dH2O stirring on a magnetic stir plate, and then brought to volume with dH2O

SODIUM ACETEATE 0.3M PH 4.8 BUFFER

Final Concentration	Amount Needed	FW or Stock Con.

0.3 sodium Acetate	50ml	0.3M Na Acetate Stock
0.3M Acetic Acid	100ml	0.3M Acetic Acid Stock

Acetic acid was added to sodium acetate stirring on a magnetic stir plate until pH was reduced to 4.8.

SODIUM BICARBONATE BUFFER (2.5 L)

Final Concentration	Amount Needed	FW or Stock Con.
151.7 M NaHCO3	31.87 g	FW 84.01

Sodium bicarbonate was added to 2 L dH2O stirring on a magnetic stir plate, and then brought to volume with dH2O. It was then gassed for 45 min with 95% O2/5% CO2.

SODIUM CARBONATE (1L)

Final Concentration	Amount Needed	FW or Stock Con.
Sodium carbonate	20g	F.W 106
0.1 N NaOH	10ml	10N NaOH Stock

Sodium carbonate and 10 ml 10 N NaOH was added to 900 ml dH2O stirring on a magnetic stir of plate, and then brought to volume with dH2O.

SODIUM CHLORIDE 5.0N (40ML)

Final Concentration	Amount Needed	FW or Stock Con.
5.0N	11.688g	FW 56.44

Sodium chloride was added to 35ml dH2O stirring on a magnetic stir plate, and then brought to volume with dH2O.

SODIUM FLOURIDE 0.5M (45ML)

Final Concentration	Amount Needed	FW or Stock Con.
0.5M	0.9448g	FW 41.99

Sodium chloride was added to 35ml dH2O stirring on a magnetic stir plate, and then brought to volume with dH2O

SODIUM POTASSIUM TARTRATE (25ML)

Final Concentration	Amount Needed	FW or Stock Con.
2% Na potassium tartrate	0.5g	FW 282.23

Sodium potassium tartrate was added to 20ml dH2O stirring on a magnetic stir plate, and then brought to volume with dH2O

SODIUM PYROPHOSPHATE 100MM (45ML)

Final Concentration	Amount Needed	FW or Stock Con.
100mM	2.0075g	FW 446.1

Sodium pyrophosphate was added to 35ml dH2O stirring on a magnetic stir plate, and then brought to volume with dH2O

SODIUM ORTHOVANADATE 100MM (30ML)

Final Concentration	Amount Needed	FW or Stock Con.
100mM	0.5517g	FW 183.9

Sodium orthovanadate was added to 20ml dH2O stirring on a magnetic stir plate, and then brought to volume with dH2O

TLC ACTIVATION BUFFER (3L)

Final Concentration	Amount Needed	FW or Stock Con.
2mM EDTA	2.98g	FW 372.2
90mM Potassium oxalate	52g	FW 184.2

Reagents were added to 2.4L dH2O stirring on a magnetic stir plate, and brought to volume with methanol.

TLC RUNNING BUFFER (1.1L)

Final Concentration	Amount Needed	FW or Stock Con.
50% $CHCL_3$	600ml	Stock 100% $CHCl_3$
25% Methanol	470ml	Stock 100% Methanol
6% NH_4OH	20ml	Stock 100% NH_4OH

Reagents were added to beaker stirring on a magnetic stir plate, and the brought to volume with dH_2O

TRIS HCL pH 7.5 1M BUFFER (45ml)

Final Concentration	Amount Needed	FW or Stock Con.
1M Tris pH 7.5	5.45 g	FW 121.1

Tris HCl was added to 30ml dH2O stirring on a magnetic stir plate, titrated with concentrated HCl and then brought to volume with dH2O.

TRIS HCL pH 7.5 100mM BUFFER (100ml)

Final Concentration	Amount Needed	FW or Stock Con.
100mM Tris	10ml	1M Tris pH 8.1 Stock

Concentrated Tris HCL buffer was added to 80ml dH2O stirring on a magnetic stir plate, then brought to volume with dH2O

APPENDIX B: STUDY 1 RAW DATA

Mouse treadmill run times expressed in minutes (*Indicates abbreviated run times due to sore feet/claws).

Group/mouse	Pre IPGTT #1	Pre IPGTT #2	Pre Perfusion #1	Pre Perfusion #2
WTX	180	180	178	170
WTX	165	170	180	160*
WTX	180	180	180	180
WTX	180	180	180	180
WTX	180	180	180	180
WTX	180	150*	180	180
WTX	180	140*	180	180
WTX	180	180	180	160
WTX	180	180	180	180
WTX	180	180	180	170
WTX	180	180	180	180
WTX	180	180	180	180

Group/mouse	Pre IPGTT #1	Pre IPGTT #2	Pre Perfusion #1	Pre Perfusion #2
TGX	180	180	180	170
TGX	180	180	180	180
TGX	180	180	180	180
TGX	170	170	180	180
TGX	180	180	180	180
TGX	180	180	180	180
TGX	160*	140*	180	160*
TGX	180	180	160*	160*
TGX	180	180	180	180
TGX	180	180	180	180
TGX	180	170	180	180
TGX	170	180	180	180
TGX	180	180	180	170

Group/mouse	Pre IPGTT #1	Pre Perfusion #1

SUPER	180	180
SUPER	180	180
SUPER	180	180
SUPER	180	180
SUPER	180	180
SUPER	180	160
SUPER	180	180
SUPER	180	180

Mixed gastrocnemius muscle glycogen expressed in µmol • g wet weight^{-1}.

WT	TG	WTX	TGX	SUPER	
13.02	244.58	18.8	149.07	149.6	
32.18	190.92	24.49	211.21	119.16	
37.45	137.19	45.81	183.67	82.86	
20.47	184.47	39.33	227.56	125.49	
14.82	297.02	22.78	190.59	90.04	
41.83	204.68	15.05	267.96	90.43	
19.81	139.15	25.34	145.74	99.5	
20.76	206.88	38.92	311.75	101.88	
51.66	186.87	44.75	294.37	101.34	
16.38	117.36	31.13	231.31	84.01	
33.95	180.42	28.54	232.95	85.77	
22.64	170.54	33.95	233.39	116.02	
48.51	249.23	32.33	311.75		
	200.46		117.38		
			167.48		
28.73	193.56	30.86	218.41	103.84	mean
3.621	12.739	2.658	15.623	5.842	SEM

Blood glucose response to an intraperitoneal injection of 10% glucose solution (1 mg • g body weight^{-1}). Values are expressed as mM.

group	pre	30min	60min	120min	150min
WT	7.11	16.00	16.56	11.22	9.94
WT	6.94	10.56	10.67	9.11	7.72
WT	6.11	15.78	17.89	13.83	9.33

WT	7.28	14.28	12.72	8.72	7.94
WT	7.83	15.33	15.22	12.56	10.72
WT	6.78	13.78	12.11	11.06	9.11
WT	7.94	13.83	16.33	15.44	10.94
WT	3.94	8.61	7.67	6.78	7.11
WT	7.11	12.11	11.33	9.89	9.11
WT	5.17	16.06	12.94	7.06	8.22
mean	6.62	13.63	13.34	10.57	9.02
SEM	0.39	0.79	0.99	0.89	0.40

group	pre	30min	60min	120min	150min
TG	7.94	15.44	17.22	10.17	8.89
TG	7.78	18.83	19.83	17.22	12.83
TG	5.61	13.50	11.06	9.50	9.17
TG	6.78	16.56	15.50	14.11	11.72
TG	6.06	14.50	12.00	9.67	8.17
TG	7.06	13.67	13.39	9.72	9.89
TG	6.33	16.00	14.17	10.22	9.17
TG	6.11	20.50	18.00	12.17	10.28
TG	5.61	14.28	14.83	11.06	8.39
TG	6.83	18.50	19.67	15.61	15.56
TG	4.61	9.50	6.83	5.11	6.17
TG	6.67	16.28	14.83	12.78	11.22
mean	6.45	15.63	14.78	11.44	10.12
SEM	0.27	0.84	1.08	0.93	0.71

group	pre	30min	60min	120min	150min
WTX	2.94	9.94	8.44	6.67	5.56
WTX	3.67	14.22	12.89	9.56	9.22
WTX	3.22	10.56	9.50	8.28	7.89
WTX	2.78	10.06	7.44	4.50	5.00
WTX	2.89	10.89	10.06	7.67	7.11
WTX	2.94	15.72	16.50	8.72	8.06
WTX	3.78	8.89	9.00	6.89	6.39
WTX	3.94	8.61	7.67	6.78	7.11
WTX	4.83	21.00	18.94	14.61	10.89
WTX	2.50	12.67	10.94	7.00	6.33
WTX	2.94	14.83	11.67	7.94	6.11
WTX	3.72	19.83	16.11	10.56	7.33

mean	3.35	13.10	11.60	8.26	7.25
SEM	0.18	1.15	1.05	0.70	0.45

group	pre	30min	60min	120min	150min
TGX	3.72	14.11	13.44	7.89	6.94
TGX	3.39	12.94	12.44	10.61	9.22
TGX	2.72	14.78	12.17	6.44	6.61
TGX	2.72	16.11	14.94	7.67	6.17
TGX	2.94	14.72	13.44	9.78	7.39
TGX	4.78	19.28	19.78	11.89	11.61
TGX	3.72	12.28	10.56	7.33	6.72
TGX	3.22	12.89	11.22	7.28	7.56
TGX	3.56	15.78	15.06	10.50	8.00
TGX	3.83	15.83	15.61	11.17	7.83
TGX	3.94	16.50	13.33	9.00	5.67
TGX	5.61	19.50	18.11	11.83	9.11
TGX	3.67	20.78	19.78	18.28	13.22
mean	3.68	15.81	14.61	9.97	8.16
SEM	0.22	0.74	0.84	0.86	0.60

group	pre	30min	60min	120min	150min
SUPER	7.00	11.94	10.39	11.00	8.11
SUPER	6.94	13.67	13.11	12.61	8.28
SUPER	5.28	12.56	13.78	13.44	9.22
SUPER	6.00	11.72	14.11	12.22	10.89
SUPER	6.44	12.22	11.11	9.00	8.11
SUPER	5.83	10.78	10.33	9.28	8.28
SUPER	6.17	10.50	11.00	9.89	9.22
SUPER	5.67	13.72	13.17	11.22	10.89
mean	6.17	12.14	12.13	11.08	9.13
SEM	0.21	0.42	0.56	0.57	0.64

Plasma insulin response to intraperitoneal injection of 10% glucose solution (1 mg • g body weight^{-1}). Values are expressed as µU • ml^{-1}.

group	pre	30 min	60 min
WT	16.18	25.39	26.89
WT	8.43	35.23	20.68
WT	9.20	29.59	18.78

WT	8.98	29.22	17.09
WT	30.00	45.90	33.63
WT	16.67	27.83	no sx.
WT	23.96	39.68	39.40
WT	14.32	29.34	19.45
WT	29.43	18.98	22.13
WT	18.96	no sx.	15.03
mean	17.61	31.24	23.68
SEM	2.535	2.658	2.709

group	pre	30 min	60 min
TG	22.33	no sx.	21.74
TG	9.77	27.39	8.06
TG	18.22	19.30	15.96
TG	8.73	26.66	9.96
TG	26.78	46.81	48.94
TG	11.35	24.06	11.65
TG	13.94	53.62	32.72
TG	7.12	17.22	20.00
TG	18.38	26.12	31.27
TG	32.92	34.26	28.93
TG	7.14	15.73	10.36
TG	12.27	23.34	24.98
mean	15.74	28.59	22.05
SEM	2.370	3.607	3.488

group	pre	30 min	60 min
WTX	3.83	3.95	5.46
WTX	4.38	4.73	8.94
WTX	3.01	0.70	5.52
WTX	2.60	0.44	4.27
WTX	0.70	0.70	2.13
WTX	0.70	0.70	0.83
WTX	1.18	9.00	3.98
WTX	0.70	12.65	6.72
WTX	9.39	13.24	10.69
WTX	2.10	2.92	0.78
WTX	0.17	4.00	1.24
WTX	0.69	7.95	0.70
mean	2.45	5.08	4.27
SEM	0.746	1.331	0.964

group	pre	30 min	60 min
TGX	0.70	13.80	3.24
TGX	6.54	12.17	5.60
TGX	0.87	16.16	4.48
TGX	0.70	11.41	7.41

TGX	1.09	12.09	4.81
TGX	3.57	7.44	4.73
TGX	0.94	16.24	5.95
TGX	0.70	21.07	7.84
TGX	11.75	10.36	2.76
TGX	0.22	4.49	7.20
TGX	0.47	4.49	4.87
TGX	2.53	6.65	6.42
TGX	0.70	20.80	0.70
mean	2.37	12.09	5.08
SEM	0.921	1.526	0.560

group	pre	30 min	60 min
SUPER	0.90	3.78	1.80
SUPER	1.01	2.16	1.80
SUPER	2.16	2.16	2.16
SUPER	11.52	12.96	11.16
SUPER	1.44	1.80	2.16
SUPER	1.44	8.10	12.96
SUPER	7.34	10.08	15.12
SUPER	6.48	12.96	12.96
SUPER	7.78	10.01	12.96
SUPER	2.16	11.16	2.16
mean	4.22	7.52	7.52
SEM	1.184	1.452	1.860

Low (0.2 mU • ml^{-1}) insulin 2-deoxyglucose uptake results expressed in µmol • g wet weight^{-1} • h^{-1}.

WT	TG	WTX	TGX	SUPER	
7.94	4.34	11.31	12.73	4.33	
6.99	4.85	9.52	7.86	2.79	
7.24	5.46	4.69	8.03	5.37	
6.87	4.12	7.77	6.34	2.82	
7.53	2.85	7.04	8.41	4.66	
7.56	5.3	7.49	8.25	4.43	
	6.12		8.2		
			10.05		
			8.65		
7.36	4.72	7.97	8.72	4.07	mean
0.163	0.404	0.921	0.593	0.426	SEM

High (10 mU • ml^{-1}) insulin 2-deoxyglucose uptake results expressed in µmol • g wet weight^{-1} • h^{-1}.

WT	TG	WTX	TGX	SUPER	
7.88	4.46	7.27	10.08	7.8	
8.26	5.69	12.75	11.22	8.03	
7.37	3.94	10.84	13.49	6.48	
12.52	6.32	6.74	9.61	7.94	
12.01	7.38	11.57	12.19	8.26	
6.78	7.88	12.01	13.72	8.19	
8.9	9.01	12.82			
9.10	6.38	10.57	11.72	7.78	mean
0.856	0.695	0.958	0.701	0.269	SEM

Low (0.2 mU • ml^{-1}) insulin glycogen synthase activity expressed in nmol • g wet weight^{-1} • min^{-1}.

WT				
25mM	1.5mM	0.5mM	.01mM	
288.57	35.33	12.20	8.10	
263.38	43.05	10.14	3.02	
135.88	117.96	20.17	10.00	
236.89	36.32	11.66	5.14	
231.18	58.17	13.54	6.57	mean
33.473	20.005	2.252	1.548	SEM

TG				
25mM	1.5mM	0.5mM	.01mM	
699.71	99.76	35.79	19.71	
864.58	150.19	55.75	24.77	
798.87	195.50	55.76	16.38	
576.22	70.88	17.64	8.58	
734.85	129.08	41.24	17.36	mean
62.800	27.544	9.165	3.397	SEM

WTX				
25mM	1.5mM	0.5mM	.01mM	
236.91	62.46	20.59	10.94	

186.55	34.81	13.78	4.80	
95.26	19.53	7.67	6.40	
161.30	22.84	8.27	3.64	
170.01	34.91	12.58	6.45	mean
29.457	9.752	3.004	1.602	SEM

TGX 25mM	1.5mM	0.5mM	.01mM	
219.21	28.17	10.89	10.20	
707.29	173.25	38.15	14.66	
630.77	119.83	38.88	8.48	
795.55	107.12	26.92	7.02	
661.07	125.78	36.17	9.75	
698.67	131.50	35.03	9.98	mean
35.921	14.452	2.763	1.657	SEM

SUPER 25mM	1.5mM	0.5mM	.01mM	
142.41	55.42	42.76	22.47	
49.75	9.44	9.91	7.97	
50.94	11.34	6.75	2.12	
33.61	2.92	2.63	0.98	
239.67	41.67	12.71	3.42	
103.276	24.158	14.95	7.39	mean
39.110	10.286	6.528	3.608	SEM

Low (0.2 mU • ml^{-1}) insulin glycogen synthase activity (I/I+D) ratio and fractional velocity (0.5 mM and 1.5 mM G-6-P expresssed as percent of maximal activity at 25 mM G-6-P).

WT I/(I+D) ratio	fract. velo. 0.5	fract. velo. 1.5	
0.028	4.23	12.24	
0.011	3.85	16.35	
0.074	14.84	86.81	
0.022	4.92	15.33	
0.03	6.96	32.68	mean

0.014	2.637	18.064	SEM

TG I/(I+D) ratio	fract. velo. 0.5	fract. velo. 1.5	
0.028	5.11	14.26	
0.029	6.45	17.37	
0.021	6.98	24.47	
0.015	3.06	12.30	
0.02	5.40	17.10	mean
0.003	0.873	2.670	SEM

WTX I/(I+D) ratio	fract. velo. 0.5	fract. velo. 1.5	
0.046	8.69	26.36	
0.026	7.39	18.66	
0.067	8.05	20.50	
0.023	5.13	14.16	
0.04	7.31	19.92	mean
0.010	0.776	2.527	SEM

TGX I/(I+D) ratio	fract. velo. 0.5	fract. velo. 1.5	
0.047	4.97	12.85	
0.021	5.39	24.49	
0.013	6.16	19.00	
0.009	3.38	13.46	
0.015	5.47	19.03	
0.01	5.10	19.00	mean
0.002	0.599	2.252	SEM

SUPER I/(I+D) ratio	fract. velo. 0.5	fract. velo. 1.5
0.158	30.03	38.92
0.160	19.92	18.97
0.042	13.25	22.26
0.029	7.83	8.69
0.014	5.30	17.39

0.06	11.57	16.83	mean
0.033	3.238	2.897	SEM

High (10 mU • ml^{-1}) insulin glycogen synthase activity expressed in nmol • g wet weight^{-1} • min^{-1}.

WT				
25mM	1.5mM	0.5mM	.01mM	
376.77	78.68	28.07	11.53	
217.62	56.55	12.73	3.73	
153.69	26.03	14.87	11.78	
143.29	17.37	11.65	5.23	
222.84	44.66	16.83	8.07	mean
53.876	14.114	3.806	2.094	SEM

TG				
25mM	1.5mM	0.5mM	.01mM	
1020.63	154.91	44.26	6.81	
1224.64	438.22	113.26	26.51	
1960.47	622.93	177.73	60.96	
2212.08	471.87	107.84	25.28	
1604.46	421.98	110.77	29.89	mean
285.929	97.664	27.267	11.294	SEM

WTX				
25mM	1.5mM	0.5mM	.01mM	
343.46	47.83	16.78	2.33	
440.68	106.48	44.30	30.75	
599.74	61.99	26.39	7.82	
147.14	60.17	19.46	4.60	
382.76	69.12	26.73	11.38	mean
94.647	12.845	6.196	6.556	SEM

TGX			
25mM	1.5mM	0.5mM	.01mM
1706.20	502.29	126.99	23.18
1378.59	504.70	144.27	28.23
1536.86	383.36	96.35	21.74

1548.52	299.74	80.07	21.36	
1545.81	311.2	80.55	17.03	
1543.20	400.26	105.65	22.31	mean
51.837	44.519	12.880	1.802	SEM

SUPER 25mM	1.5mM	0.5mM	.01mM	
231.04	35.69	13.77	8.88	
86.01	24.72	19.71	16.94	
73.34	14.93	15.32	10.28	
304.00	42.01	11.85	7.64	
139.80	15.76	3.36	2.00	
166.84	26.62	12.80	9.15	mean
44.126	5.373	2.693	2.402	SEM

High (10 mU • ml^{-1}) insulin glycogen synthase activity (I/I+D) ratio and fractional velocity (0.5 mM and 1.5 mM G-6-P expresssed as percent of maximal activity at 25 mM G-6-P).

WT I/(I+D) ratio	fract. velo. 0.5	fract. velo. 1.5	
0.031	7.45	20.88	
0.017	5.85	25.99	
0.077	9.68	16.94	
0.036	8.13	12.12	
0.04	7.78	18.98	mean
0.013	0.793	2.943	SEM

TG I/(I+D) ratio	fract. velo. 0.5	fract. velo. 1.5	
0.007	4.34	15.18	
0.022	9.25	35.78	
0.031	9.07	31.77	
0.011	4.88	21.33	
0.02	6.88	26.02	mean
0.005	1.319	4.726	SEM

WTX I/(I+D)	fract. velo.	fract. velo.

ratio	0.5	1.5	
0.007	4.89	13.93	
0.070	10.05	24.16	
0.013	4.40	10.34	
0.031	13.23	40.89	
0.03	8.14	22.33	mean
0.014	2.123	6.846	SEM

TGX I/(I+D) ratio	fract. velo. 0.5	fract. velo. 1.5	
0.014	7.44	29.44	
0.020	10.47	36.61	
0.014	6.27	24.94	
0.014	5.17	19.36	
0.011	5.21	20.13	
0.01	6.78	25.26	mean
0.002	1.255	3.980	SEM

SUPER I/(I+D) ratio	fract. velo. 0.5	fract. velo. 1.5	
0.038	5.96	15.45	
0.197	22.92	28.74	
0.140	20.89	20.36	
0.025	3.90	13.82	
0.014	2.40	11.27	
0.09	12.53	18.55	mean
0.045	5.438	3.899	SEM

Low (0.2 mU • ml^{-1}) insulin IRS-1-associated PI3-kinase results expressed as percent of insulin-stimulated mouse gastrocnemius standard.

WT	TG	WTX	TGX
1.38	2.66	2.82	4.98
0.69	1.56	0.97	1.78
1.52	1.39	1.84	1.35
0.59	1.57	0.94	2.15
0.73	1.52	2.03	2.06
			1.20

0.98	1.74	1.72	2.25	mean
0.194	0.232	0.353	0.567	SEM

High (10 mU • ml^{-1}) insulin IRS-1-associated PI3-kinase results expressed as percent of insulin-stimulated mouse gastrocnemius standard.

WT	TG	WTX	TGX	SUPER	
2.13	2.99	4.37	6.69	1.58	
2.35	0.91	7.44	0.82	0.75	
1.70	3.77	7.26	1.95	1.91	
3.19	2.20	2.18	3.86	1.44	
2.51	2.35	3.14	2.47	1.09	
	5.78		2.52		
2.38	3.00	4.88	3.05	1.35	mean
0.245	0.677	1.068	0.831	0.200	SEM

Low (0.2 mU • ml^{-1}) insulin total PKB/Akt results expressed as percent of insulin-stimulated mouse gastrocnemius standard.

WT	TG	WTX	TGX	SUPER	
48.62	56.82	42.33	68.02	67.45	
65.12	62.70	45.00	47.21	69.21	
38.90	62.68	50.81	52.85	62.88	
35.83	61.68	48.08	68.89	68.45	
37.23	68.41	69.08	65.21	72.37	
42.85	69.89	65.83	39.24	43.79	
	50.13	38.76	36.93		
		36.34	37.57		
			44.09		
44.76	61.76	49.53	51.11	64.02	mean
4.487	2.543	4.255	4.400	4.237	SEM

High (10 mU • ml^{-1}) insulin total PKB/Akt results expressed as percent of insulin-stimulated mouse gastrocnemius standard.

WT	TG	WTX	TGX	SUPER

59.42	63.21	70.05	108.02	63.31	
61.47	65.24	117.78	115.69	83.83	
75.62	65.99	54.14	52.48	88.38	
65.31	38.05	48.00	67.48	87.91	
51.27	66.00	48.43	38.05	91.08	
	67.91	38.57	36.81		
	49.88	48.95	58.19		
	42.48	45.67			
62.62	57.34	58.95	68.10	82.90	mean
3.979	4.241	8.993	12.04	5.032	SEM

Low (2 mU • ml^{-1}) insulin phosphorylated PKB/Akt (Ser 473) results expressed as percent of insulin-stimulated mouse gastrocnemius standard.

WT	TG	WTX	TGX	SUPER	
48.62	70.75	62.41	84.42	32.80	
33.28	36.37	42.84	56.98	15.00	
60.20	46.83	63.63	68.26	31.73	
41.41	32.53	78.50	108.43	29.27	
21.98	53.78	52.14	100.75	28.93	
23.28	38.82	91.10	81.16	38.09	
	66.93	65.85	63.15		
		47.81	55.23		
			48.69		
31.13	49.43	63.04	74.12	29.30	mean
6.090	5.680	5.661	6.966	3.163	SEM

High (10 mU • ml^{-1}) insulin phosphorylated PKB/Akt (Ser 473) results expressed as percent of insulin-stimulated mouse gastrocnemius standard.

WT	TG	WTX	TGX	SUPER
76.83	54.58	140.77	85.99	18.94
52.00	43.93	99.41	62.34	44.83
42.13	70.73	69.10	57.93	42.53
78.00	60.66	67.08	104.46	22.61
55.15	81.88	67.90	90.72	11.55
	115.15	25.86	43.43	

	87.54	79.17	58.68		
	25.57	78.92			
60.82	67.51	78.53	71.94	28.09	mean
7.109	9.835	11.509	8.288	6.618	SEM

Total protein content in mixed gastrocnemius muscle expressed as mg protein • g muscle wet weight^{-1}.

WT	239.86	TG	232.27
WT	245.45	TG	180.45
WT	153.63	TG	274.34
WT	259.52	TG	193.05
WT	267.65	TG	276.08
WT	208.46	TG	150.87
WT	239.86	TG	240.71
WT	226.89	TG	253.05
WT	259.52	TG	206.08
WT	267.65	TG	180.87
WTX	240.03	TG	200.21
WTX	254.34	TG	203.66
WTX	227.81	TGX	285.88
WTX	163.93	TGX	175.53
WTX	180.65	TGX	189.60
WTX	101.62	TGX	244.18
WTX	199.85	TGX	196.68
WTX	202.55	TGX	185.16
WTX	293.88	TGX	220.23
WTX	178.44	TGX	189.11
WTX	187.22	TGX	201.02
WTX	233.95	TGX	187.74
WTX	190.36	TGX	230.60
SUPER	265.72	TGX	165.11
SUPER	184.49	TGX	224.36
SUPER	118.28	mean	210.63
SUPER	165.42	SEM	4.765
SUPER	194.28		
SUPER	259.34		

SUPER	225.51
SUPER	209.63
SUPER	173.71
SUPER	175.01
SUPER	212.93
mean	210.10
SEM	2.111

GLUT4 protein content in mixed gastrocnemius muscle expressed as percent of heart standard.

WT	36.06
WT	85.23
WT	54.57
WT	40.06
WT	47.04
WT	54.26
WT	69.93
WT	70.91
WT	72.85
WT	49.70
mean	58.06
SEM	5.043
WTX	93.99
WTX	58.23
WTX	74.08
WTX	32.87
WTX	46.01
WTX	79.56
WTX	63.39
WTX	82.19
WTX	64.22
WTX	40.22
mean	63.48
SEM	6.203

TG	31.26
TG	46.33
TG	74.56
TG	53.05
TG	76.08
TG	50.87
TG	60.71
TG	78.62
TG	52.02
TG	77.91
TG	89.35
TG	78.66
mean	64.12
SEM	5.04
TGX	85.88
TGX	75.53
TGX	89.60
TGX	44.18
TGX	96.68
TGX	85.16
TGX	87.74
TGX	70.60
TGX	65.11
TGX	54.36
mean	75.48

SUPER	76.45	SEM	5.331
SUPER	86.44		
SUPER	67.35	TG mean	69.28
SUPER	79.76	TG SEM	3.784
SUPER	81.9		
SUPER	77.85		
SUPER	83.56		
mean	79.04		
SEM	2.333		
WT mean	65.51		
WT SEM	3.364		

Hexokinase activity in mixed gastrocnemius muscle expressed as µmol • g • min^{-1}.

WT	3.77	TG	4.47
WT	2.14	TG	3.21
WT	2.25	TG	3.55
WT	5.06	TG	2.86
WT	2.24	TG	4.49
WT	3.51	TG	3.06
WT	2.80	TG	3.20
WT	3.06	TG	3.35
WT	3.18	TG	3.85
mean	3.11	TG	3.74
SEM	0.310	TG	4.47
		TG	3.76
WTX	2.95	TG	3.16
WTX	2.03	TG	3.83
WTX	2.23	TG	2.62
WTX	3.93	mean	3.57
WTX	3.18	SEM	0.151
WTX	1.36		
WTX	2.91	TGX	3.15
WTX	2.90	TGX	3.59
WTX	2.64	TGX	4.22

WTX	2.08
WTX	2.87
WTX	3.18
WTX	2.12
WTX	3.34
WTX	2.06
WTX	1.94
mean	2.61
SEM	0.166
SUPER	4.12
SUPER	4.45
SUPER	0.86
SUPER	3.68
SUPER	1.61
SUPER	1.60
SUPER	3.74
SUPER	1.83
mean	2.53
SEM	0.195

TGX	3.00
TGX	2.28
TGX	3.02
TGX	3.20
TGX	3.60
TGX	3.26
TGX	2.71
TGX	1.96
TGX	3.13
TGX	3.60
TGX	2.60
TGX	4.27
TGX	2.73
mean	3.14
SEM	0.157

APPENDIX C: STUDY 2 RAW DATA

Study 2: Isolated, *in situ* whole gastrocnemius peak twitch tension (P_t expressed in mN), wet weight (ww), muscle length (ml), and peak tetanic tension (P_o expressed in mN).

group	P_t	ww(mg)	ml(mm)	P_o
WT	1030	100	18	3580
WT	1185	130.4	18	3553
WT	1080	135.2	17	3444
WT	1043	100	18	3185
WT	1030	104	16	3061
WT	753	99.5	14	3003
TG	1148	121	15	2901
TG	987	90	16	2246
TG	1092	101	16	2462
TG	1228	155	19	3172
TG	1030	98.7	15	2475
TG	1123	141	20	3320
TG	1148	161	14	3395

Study 2: Isolated, *in situ* whole gastrocnemius tension assessed during electrical stimulation over 30 min. Values are expressed as mN.

group	5min	10min	15min	20min	25min	30min	avg
WT	1413	1388	1358	1358	1364	1283	1360.67
WT	1524	1530	1604	1617	1635	1581	1581.83
WT	1655	1623	1679	1629	1635	1623	1640.67
WT	1462	1506	1580	1598	1753	1666	1594.17
WT	1771	1543	1728	1841	1851	1691	1737.50
WT	1523	1675	1498	1576	1602	1534	1568.00
						mean	1580.47
						SEM	50.643
TG	1938	1925	1932	1876	1882	1851	1900.67
TG	1555	1617	1586	1592	1574	1567	1581.83
TG	1728	1629	1685	1612	1650	1650	1659.00
TG	1555	1475	1456	1401	1419	1432	1456.33
TG	1808	1790	1833	1833	1839	1827	1821.67
TG	1635	1771	1545	1450	1620	1580	1600.17
TG	2179	2358	2160	1777	1851	2000	2054.17
						mean	1724.83

	SEM	78.857

Study 2: Isolated, *in situ* whole gastrocnemius tension assessed during electrical stimulation over 30 min and expressed as a percentage of peak tetanic tension.

group	5min	10min	15min	20min	25min	30min	avg
WT	39.5	38.8	37.9	37.9	38.1	35.8	38.0
WT	42.9	43.1	45.1	45.5	46.0	44.5	44.5
WT	48.1	47.1	48.8	47.3	47.5	47.1	47.6
WT	45.9	47.3	49.6	50.2	55.0	52.3	50.1
WT	57.9	50.4	56.5	60.1	60.5	55.2	56.8
WT	50.72	55.78	49.88	52.48	53.35	51.08	52.21
						mean	48.20
						SEM	2.650
TG	66.8	66.4	66.6	64.7	64.9	63.8	65.5
TG	69.2	72.0	70.6	70.9	70.1	69.8	70.4
TG	70.2	66.2	68.4	65.5	67.0	67.0	67.4
TG	49.0	46.5	45.9	44.2	44.7	45.1	45.9
TG	73.1	72.3	74.1	74.1	74.3	73.8	73.6
TG	49.2	53.3	46.5	43.7	48.8	47.6	48.2
TG	64.2	69.5	63.6	52.3	54.5	58.9	60.5
						mean	61.6
						SEM	4.076

Study 2: *In situ* electrical stimulation data from isolated whole gastrocnemius. Muscle peak tetanic tension (P_o), optimal length (L_o), specific gravity, fiber/muscle length ratio, estimated cross-sectional area (CSA), and specific force are presented.

group	P_o(N)	ML_o(cm)	MW(g)	Sp. Gravity (g/cm^3)	fiber/muscle length ratio	CSA(cm^2)	Sp. Force (N/cm^2)	
WT	3.580	1.8	0.100	1.06	0.4	0.13103	27.32	
WT	3.553	1.8	0.130	1.06	0.4	0.17086	20.79	
WT	3.444	1.7	0.135	1.06	0.4	0.18757	18.36	
WT	3.185	1.8	0.100	1.06	0.4	0.13103	24.31	
WT	3.061	1.6	0.104	1.06	0.4	0.15330	19.97	
WT	3.003	1.4	0.100	1.06	0.4	0.16762	17.92	
	3.30	1.68	0.11	1.06	0.40	0.16	21.44	mean
	0.104	0.065	0.007	0.000	0.000	0.009	1.498	SEM
TG	2.901	1.4	0.121	1.06	0.4	0.2038	14.23	

TG	2.246	1.6	0.090	1.06	0.4	0.1327	16.93	
TG	2.462	1.6	0.101	1.06	0.4	0.1489	16.54	
TG	3.172	1.9	0.155	1.06	0.4	0.1924	16.49	
TG	2.475	1.5	0.099	1.06	0.4	0.1552	15.95	
TG	3.320	2.0	0.141	1.06	0.4	0.1663	19.97	
TG	3.395	1.4	0.161	1.06	0.4	0.2712	12.52	
	2.85	1.63	0.12	1.06	0.40	0.18	16.09	mean
	0.175	0.089	0.011	0.000	0.000	0.018	0.877	SEM

Study 2: *In situ* electrical stimulation data from isolated whole gastrocnemius. Muscle peak tetanic tension (P_o) and percent of Po throughout 30 min stimulation period.

		Percent of Po						
group	P_o(N)	5 min	10 min	15 min	20 min	25 min	30 min	Avg.	
WT	3.580	39.47	38.77	37.93	37.93	38.10	35.84	38.01	
WT	3.553	42.89	43.06	45.14	45.51	46.02	44.50	44.52	
WT	3.444	48.05	47.13	48.75	47.30	47.47	47.13	47.64	
WT	3.185	45.90	47.28	49.61	50.17	55.04	52.31	50.05	
WT	3.061	57.86	50.41	56.45	60.14	60.47	55.24	56.76	
WT	3.003	50.72	55.78	49.88	52.48	53.35	51.08	52.21	
	3.30	47.48	47.07	47.96	48.92	50.07	47.68	48.20	mean
	0.104	2.622	2.394	2.501	3.029	3.215	2.834	2.650	SEM
TG	2.901	66.80	66.36	66.60	64.67	64.87	63.81	65.52	
TG	2.246	69.23	71.99	70.61	70.88	70.08	69.77	70.43	
TG	2.462	70.19	66.17	68.44	65.48	67.02	67.02	67.38	
TG	3.172	49.02	46.50	45.90	44.17	44.74	45.15	45.91	
TG	2.475	73.05	72.32	74.06	74.06	74.30	73.82	73.60	
TG	3.320	49.25	53.34	46.54	43.67	48.80	47.59	48.20	
TG	3.395	64.18	69.46	63.62	52.34	54.52	58.91	60.51	
	2.85	63.10	63.73	62.25	59.32	60.62	60.87	61.65	mean
	0.175	3.754	3.756	4.317	4.732	4.268	4.142	4.076	SEM

Study 2: 2-Deoxyglucose uptake from mixed gastrocnemius muscle during hindlimb perfusion with 0.2 µU • ml^{-1} insulin. Values are expressed as µmol • g^{-1} • h^{-1}.

WT non-stim	WT stim	TG non-stim	TG stim
2.84	7.13	2.44	8.60
2.02	7.27	3.41	7.89

3.31	5.16	4.33	9.15	
4.15	7.27	4.18	7.29	
5.78	7.36	3.74	7.34	
5.31	7.79	3.66	7.04	
3.90	7.00	3.63	7.89	mean
0.594	0.379	0.275	0.340	SEM

Study 2 (Incorporation study): Mixed gastrocnemius muscle glycogen content determined by a modified method of Lo et al. (1970) expressed as µmol • g wet weight^{-1}.

WT non-stim	WT stim	TG non-stim	TG stim	
32.12	17.61	282.57	222.05	
25.65	6.08	233.36	213.04	
32.33	25.18	193.64	120.27	
37.66	23.63	239.16	138.09	
34.73	29.84	212.64	150.69	
29.20	12.18			
23.01	9.38			
47.51	18.53			
47.37	16.64			
34.40	17.67	232.18	168.83	mean
2.871	2.569	14.959	20.517	SEM

Study 2 (Incorporation study): [^{14}C] Glucose incorporation in mixed gastrocnemius muscle during hindlimb perfusion with 0.2 µU • ml^{-1} insulin. Values are expressed as µmol • g^{-1} • h^{-1}.

WT non-stim	WT stim	TG non-stim	TG stim	
0.07	0.17	1.28	2.61	
0.85	0.97	1.80	3.62	
0.67	0.87	0.58	2.82	
0.61	1.22	1.34	2.42	
0.48	1.29	2.89	3.95	
0.87	1.75			
1.07	1.79			
0.41	1.34			
0.62	1.43			
0.63	1.20	1.58	3.08	mean

0.098	0.165	0.382	0.298	SEM

Study 2: Low (0.2 mU • ml^{-1}) insulin glycogen synthase activity expressed as nmol • g wet weight^{-1} • min^{-1}.

WT STIM. 25 mM	1.5 mM	0.5 mM	0.1 mM	
67.40	28.45	22.75	34.63	
82.81	26.93	27.98	20.67	
198.69	49.06	29.32	23.90	
68.57	63.84	51.55	34.90	
38.18	32.76	22.37	16.69	
165.47	15.76	2.84	1.37	
103.52	36.13	26.14	22.03	mean
25.897	7.083	6.397	5.116	SEM

WT NON-STIM. 25 mM	1.5 mM	0.5 mM	0.1 mM	
49.15	17.69	18.34	13.58	
54.61	31.5	22.89	20.87	
44.17	25.06	23.83	22.45	
71.26	58.31	50.61	45.62	
101.66	25.19	6.56	1.48	
64.17	31.55	24.45	20.80	mean
10.423	7.038	7.227	6.596	SEM

TG STIM. 25 mM	1.5 mM	0.5 mM	0.1 mM	
1547.77	254.26	84.62	36.38	
1835.94	337.25	100.78	41.46	
1518.2	225.04	87.04	63.45	
2144.08	350.26	85.11	22.05	
2584.51	666.22	152.96	29.46	
1926.10	366.61	102.10	38.56	mean
199.833	78.600	13.057	7.029	SEM

TG NON-STIM. 25 mM	1.5 mM	0.5 mM	0.1 mM
493.5	126.98	63.08	33.62

993.24	160.21	53.55	25.06	
802.12	172.88	89.07	68.93	
1158.24	138.31	55.55	8.55	
713.59	129.27	34.27	8.01	
832.14	145.53	59.10	28.83	mean
114.439	9.012	8.871	11.158	SEM

Study 2: Low (0.2 mU • ml^{-1}) insulin glycogen synthase activity (I/I+D) ratio and fractional velocity (0.5 mM and 1.5 mM G-6-P expresssed as percent of maximal activity at 25 mM G-6-P).

WT STIM. I/(I+D) ratio	fract. velo. 0.5	fract. velo. 1.5	
0.51	33.75	42.21	
0.25	33.79	32.52	
0.12	14.76	24.69	
0.51	75.18	93.10	
0.44	58.59	85.80	
0.01	1.72	9.52	
0.31	36.30	47.98	mean
0.087	11.078	13.857	SEM

WT NON-STIM. I/(I+D) ratio	fract. velo. 0.5	fract. velo. 1.5	
0.28	37.31	35.99	
0.38	41.92	57.68	
0.51	53.95	56.74	
0.64	71.02	81.83	
0.01	6.45	24.78	
0.36	35.19	43.09	mean
0.097	11.128	11.569	SEM

TG STIM. I/(I+D) ratio	fract. velo. 0.5	fract. velo. 1.5
0.02	5.47	16.43
0.02	5.49	18.37
0.04	5.73	14.82

0.01	3.97	16.34	
0.01	5.92	25.78	
0.02	5.32	18.35	mean
0.006	0.347	1.941	SEM

TG NON-STIM. I/(I+D) ratio	fract. velo. 0.5	fract. velo. 1.5	
0.07	12.78	25.73	
0.03	5.39	16.13	
0.09	11.10	21.55	
0.01	4.80	11.94	
0.01	4.80	18.12	
0.04	6.56	15.83	mean
0.014	1.859	3.447	SEM

Study 2: Low (0.2 mU • ml^{-1}) insulin total and phosphorylated PKB/Akt expressed as percent of insulin-stimulated mouse gastrocnemius standard.

	Total Akt	Phophorylated	
WT	51.48	39.46	
Non-stimulated	86.24	25.14	
	77.00	43.98	
	89.18	32.60	
	93.51	46.84	
	93.24	18.98	
	81.77	34.50	mean
	6.543	4.472	SEM

	Total Akt	Phophorylated	
WT	84.67	70.44	
Stimulated	87.43	107.37	
	84.08	131.36	
	74.50	46.25	
	91.03	77.35	
	82.00	44.85	
	83.95	79.60	mean
	2.280	13.970	SEM

	Total Akt	Phophorylated	
TG	88.39	104.71	
Non-stimulated	92.38	105.57	
	92.63	142.75	
	61.93	115.00	
	60.43	62.32	
	49.99	114.88	
	74.29	107.54	mean
	7.740	10.660	SEM

	Total Akt	Phophorylated	
TG	84.60	91.20	
Stimulated	94.26	93.71	
	90.69	128.15	
	88.80	135.37	
	85.31	108.98	
	88.33	169.71	
	88.66	121.19	mean
	1.450	12.120	SEM

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